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Dimensions of the Notochord and Somites in Embryos of *Xenopus laevis* Treated with Thiocyanate

by SILVIO RANZI *and* GIANNA GAVAROSI[1]

From the Department of Zoology, University of Milano

As a result of previous research, Ranzi & Tamini (1939) pointed out that the notochord of Amphibian embryos of several species treated with sodium thiocyanate (NaSCN) solutions is larger than the notochord of control embryos. Later, several substances were identified which cause enlargement of the notochord: full data and a complete review of the literature on this subject are given by Ranzi & Citterio (1954) and Ranzi (1957). A careful statistical study on the number of nuclei in *Rana esculenta* embryos was carried out by Corti (1950). In embryos which had been treated for 24 hours with 0·05 M NaSCN, from the late blastula stage onwards, she found 1233 ± 28 nuclei at the tail-bud stage, whereas the controls reared in spring-water had only 1107 ± 24 nuclei: these two sets of data are significantly different (standard error of the difference ± 37).

Enlargement and induction of notochord by NaSCN treatment have been reported by Badínez, Carrasco, & Manríquez (1954). Notochord can be obtained from ventral explants and from explants of presumptive mesoderm by means of urea, which acts like NaSCN (Leone, 1952), or by means of NaSCN (Ôgi, 1957*a*). However, Ôgi (1957*b*) counted the nuclei of a few embryos of *Bufo vulgaris* and *R. nigromaculata* treated with NaSCN and NaI and was unable to demonstrate an increase.

For these reasons we thought it useful to repeat the work of Corti, but on another species, *Xenopus laevis* (Daudin), which appeared to be very sensitive to substances causing alterations of development (for the effect of LiCl see Bäckström, 1954).

METHODS

Eggs laid naturally by a couple of *X. laevis* were used; about 93 per cent. of the eggs were fertilized and all the controls developed normally. The eggs of this species appear to be particularly sensitive to NaSCN; a 0·01 M solution produced the familiar malformations (see Ranzi, Tamini, & Storari Offer, 1946) and yet allowed the embryos to survive.

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A 1 M NaSCN solution was made up and diluted 1 to 100 with spring-water; *Xenopus* embryos at stage 8/9 (late blastula) of Nieuwkoop (1956) were placed in the solution at 15° C. and allowed to remain there for 2 hours, i.e. during the first part of gastrulation. Then they were carefully washed with spring-water and allowed to develop in spring-water.

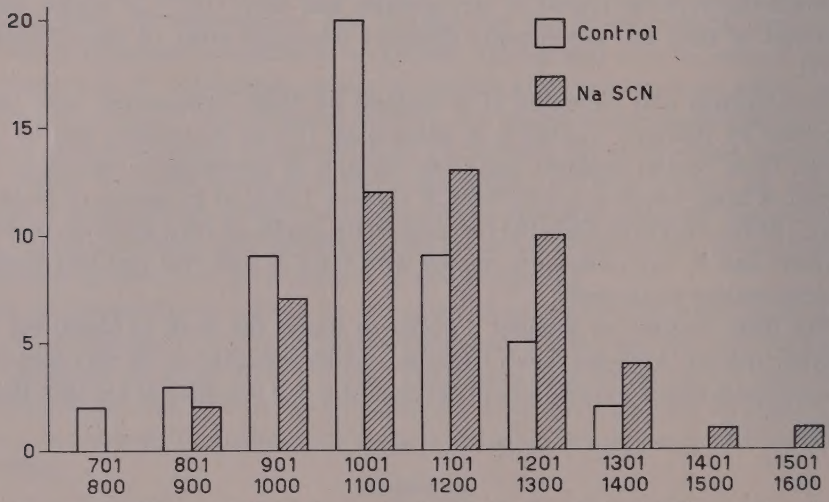
The controls were reared in identical finger bowls in spring-water.

When the control and experimental embryos at the age of 48 hours reached the tail-bud stage, 50 controls and 50 experimental embryos were fixed. During development we noticed a slight retardation in the controls compared with the NaSCN-treated embryos.

The embryos were fixed in Bouin's fluid, paraffin embedded, and sectioned at $10\,\mu$; sections were stained with Ehrlich haematoxylin and eosin. The counting of nuclei was performed with a micrometer disk mounted in a ×7 ocular and a ×62 objective. All the nuclei which appeared in one section were counted; the error in counting twice those nuclei which traversed two sections was then corrected.

RESULTS

We first measured the volume of notochord in the controls and the NaSCN-treated embryos. The volumes expressed in $\mu^3 \times 10^6$ (mean and standard error) were $11{\cdot}5 \pm 0{\cdot}59$ and $10{\cdot}0 \pm 0{\cdot}52$ respectively. The difference is not significant.

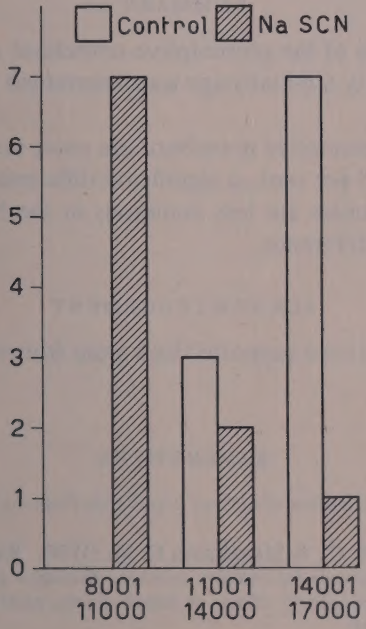


TEXT-FIG. 1. Diagram of the crude counts of nuclei in the notochord of 50 controls and of 50 embryos treated with sodium thiocyanate (NaSCN).

In comparing the numbers of nuclei we have avoided any error due to an accelerated development induced by NaSCN for the controls used were, in fact, a little more advanced than the treated embryos, and many of them showed the beginning of vacuolization in notochordal cells.

The results of the nuclear counts are given in Text-fig. 1. The mean number of nuclei of the 50 individuals in each group is: control 1056·6±18·5; NaSCN-treated 1137·3±21·5. These values must be corrected, taking into consideration the dimension of nuclei (see Abercrombie, 1946). The diameters of the nuclei are 14·1±0·72 μ for the controls and 13·2±0·67 μ for the treated embryos. Since the thickness of the sections was 10 μ, the corrected number of nuclei is therefore as follows: controls 438·4±7·67; treated embryos 490·2±9·25. There is here a highly significant difference ($0{\cdot}01 > P > 0{\cdot}001$), the increase amounting to 11·8 per cent.

We can therefore determine the ratio $\frac{\text{volume of notochord}}{\text{number of nuclei}}$, i.e. the mean volume of a cell (cells were assumed to be uninucleate). The volume, expressed in μ^3 was: controls 26,232±1220, treated embryos 20,400±567. The higher standard error of the controls is due to the fact that some embryos had larger notochordal cells due to greater vacuolization.



TEXT-FIG. 2. Diagram of the crude counts of nuclei in the somites of 10 controls and of 10 embryos treated with sodium thiocyanate (NaSCN).

Since research carried out by Cigada (1957) showed that NaSCN stimulated the division of *Amoeba*, the question arose as to whether the increase of nuclei in the notochord was a specific phenomenon, confined to this organ, or a general phenomenon detectable in all the rudiments of the embryo. Unpublished work by Ranzi & Tamini had already shown that the nuclei of the ectoderm of NaSCN-treated embryos do not increase in number. In the present investigation we examined the number of nuclei in the somites, from whose presumptive material Ranzi & Tamini (1940) had shown that notochordal cells segregate. The results are shown in Text-fig. 2. The mean number of nuclei of the 10 individuals in each group is: control 13,750 ± 565; NaSCN-treated 10,987 ± 586.

The mean diameter of the nuclei of the controls was $7{\cdot}9 \pm 0{\cdot}37\,\mu$, that of the treated embryos $7{\cdot}5 \pm 0{\cdot}36\,\mu$, and the thickness of the sections $10\,\mu$. The corrected number of nuclei is therefore: controls 7793·4 ± 315·8 nuclei; NaSCN-treated embryos 6273·3 ± 334·7 nuclei. This represents a highly significant difference ($0{\cdot}01 > P > 0{\cdot}001$). The decrease of the nuclear number in the somites of the NaSCN-treated embryos is 24·1 per cent.

The conclusion to be drawn is that cells of the somite rudiment are transformed into notochord.

SUMMARY

The number of nuclei of the presumptive notochord and somite cells of embryos of *X. laevis* at early tail-bud stage was determined in normal and NaSCN-treated embryos.

The nuclei of the presumptive notochord are more numerous in the NaSCN-treated embryos by 11·8 per cent., a significant difference.

The nuclei of the somites are less numerous in the NaSCN-treated by 24·1 per cent., a significant difference.

ACKNOWLEDGEMENT

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(Manuscript received 5 : viii : 58)

Experiments on the ‘Overgrowth’ Phenomenon in the Brain of Chick Embryos

by HARRY BERGQUIST[1]

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WITH ONE PLATE

INTRODUCTION

PATTEN (1952) described ‘a curious distortion of the central nervous system’ in human embryos measuring 5, 7, 12·5, 20, and 30 mm. in length, as well as in some pig embryos. The malformation was called ‘overgrowth of the neural tube’. Instead of the indecisive word ‘overgrowth’ the present writer suggests the more exact term ‘hypermorphosis’ should be used for this malformation. Patten described it in the following way: ‘the neural tube epithelium had started to grow wildly so that it became folded, and refolded on itself, as if it was crowded into a cranial space fairly normal in size and shape’. The phenomenon was most distinctly developed in the rostral part of the neural tube. In some cases the cranial cavity was expanded by the process, giving rise to a high-crowned skull. In other cases an encephalocoel was formed. In later papers (1953, 1957) Patten discussed this phenomenon further.

A malformation in the chick brain, similar to that described by Patten, was obtained by Källén (1955) as the result of operations in the rostral part of the rhombencephalon.

Other papers have been published on similar malformations. The literature is reviewed by Kappers (1956, 1957), who described overgrowth in a human embryo 7 weeks of age which had been damaged by a rubeola infection of its mother. The folding process was especially marked in the telencephalon, and similar abnormalities were seen in the dorsal parts of the diencephalon, mesencephalon, and rhombencephalon. The optic vesicles and the ventral parts of the brain were only slightly affected. A cranioschisis and encephaloschisis were present. In the chorioid plexus Kappers described abnormalities consisting of rosette-like cell formations. He is of the opinion that the encephalocoel was secondary to the strongly marked folding of the brain-wall.

Van Limborgh (1956) found pronounced overgrowth in the hemispheres, the

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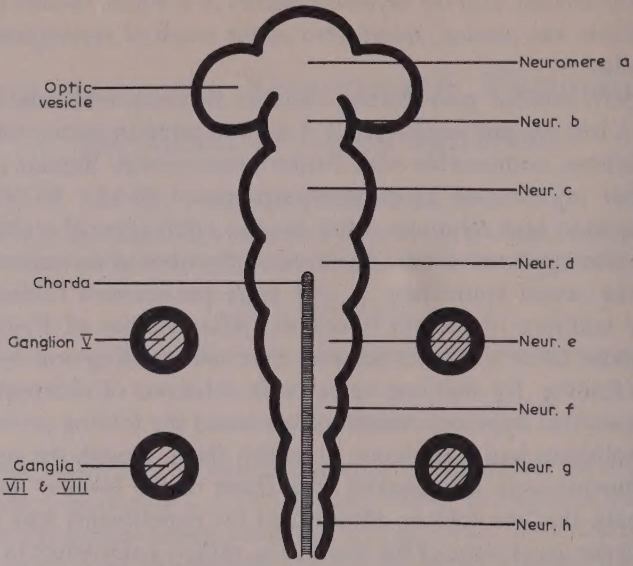
mesencephalic tectum, and the myelencephalon in a 9-mm. human embryo. An encephaloschisis was present, interpreted as the result of incomplete closure of the neural folds.

Sjodin (1957) studied post-mortem changes in chick embryos. He demonstrated that a low oxygen tension for 2–3 days resulted in strong folding of the neural epithelium, comparable with Patten's overgrowth. Similar results were obtained after injection of 2,3-dimercaptopropanol (BAL), KCN, $HgCl_2$, or indolacetic acid in high dilutions. After *in vitro* cultivation of embryos similar phenomena also appeared. Sjodin found no indications of an increased mitotic activity in the neural epithelium. A still more pronounced folding could be obtained by addition of growth inhibitors. After studies of Feulgen-stained material, Sjodin came to the conclusion that cell swelling and lysis was the basis of the folding. By studying embryos in solutions of different tonicity it could be shown that hypotonic solutions increased the folding processes, while hypertonic solutions had a tendency to inhibit them, though the results of the latter experiments were less marked than those of the former. Summing up, Sjodin remarks that the folding obtained in his experiments was no 'case of teratogeny in the usual sense of the word. It is, rather, a side effect in the morbid process.' He thinks there is a similar basis for the formation of Patten's overgrowth malformations.

MATERIAL AND METHODS

Operations were performed on chick embryos, incubated for 36–45 hours, at stage 11–14 (Hamburger & Hamilton, 1951). After opening of the egg-shells and vital staining with neutral red of the embryos, an operation was performed with glass needles of the Spemann type or with vibrating steel needles (Drury, 1941). After operation the shell openings were closed and the eggs were incubated for another 2–3 days. The age at fixation was thus 4–4½ days. The normal and operated embryos were fixed in Bouin's fluid, sectioned transversely at 10 μ and stained with Delafield's haematoxylin and eosin. A total of 28 wax-plate reconstructions and 48 graphic reconstructions have been made.

In the main experimental series of 55 specimens (4 of them from the material published by Källén, 1955) neuromere *d*, together with the underlying notochord tip, was extirpated (see Text-fig. 1; for the terminology of the neuromeres, see Bergquist, 1956). In the control series of embryos other neuromeres were extirpated: neuromere *a* in 5 specimens, neuromere *b* in 1 specimen; neuromere *c* in 7 specimens; neuromere *e* with underlying notochord in 9 specimens (including 1 of Källén's); neuromere *f* with underlying notochord in 6 specimens (including 1 of Källén's). Studies have also been made on specimens prepared by Hugosson (1957) after operations on the mesencephalon and on the most rostral part of the spinal cord; and on various normal embryos of Hamburger & Hamilton stages 21–29 belonging to the collection of the Tornblad Institute.



TEXT-FIG. 1. Schematic drawing of the neural tube of a chick embryo in dorsal view, roughly corresponding to Hamburger & Hamilton's stage 12 (incubation age approx. 43 hours); also cf. Bergquist (1956). The levels of operation are shown.

RESULTS

Normal embryos

In the normal chick brain no foldings exist which could be misinterpreted as overgrowth. The surface of the hemispheres is smooth and so is that of the mesencephalon. In the epiphysis, the rest of the diencephalic roof, the hypothalamus, and the rhombencephalon no signs of foldings similar to those described as overgrowth phenomena can be seen.

Experimental material

After extirpation of neuromere *d*, the gap in the brains of the surviving embryos was often bridged by fusion of the remnants of the brain-stem. Usually there was a thin string of nervous tissue, but in other cases the two ends fused directly. In a few cases no fusion occurred and the two ends remained separated. The location of the operation can always be seen clearly in the sections. Abnormal foldings appeared both in the hemispheres and in the mesencephalon: in 38 specimens these overgrowths were strongly marked (4 specimens having encephaloschisis in the optic tectum, and 6 having overgrowths in the optic stalks), in 5 specimens they were moderate, in 7 specimens they were faint, and in 5 specimens there were none. In some cases the roof of the diencephalon with the epiphysis was folded, and in a few cases signs of abnormal folds could be seen in the caudal parts of the hypothalamus. The morphology of these abnormalities are very similar to those described as overgrowth by Patten in human embryos.

The intention in extirpating neuromere *d* was to remove the notochordal tip. That this was done successfully was seen in the serial sections, which showed that a smaller or larger portion of the tip of the notochord was absent. In other cases the rostral end of the notochord was split into two or was club-shaped. A combination of a split and a club ending has also been seen. In one case the end was connected with the rest of the notochord by only a thin string. A complete separation of the tip from the rest of the notochord has also been observed.

In most cases the overgrowth was strongly marked in the hemispheres (Plate, fig. A). In some cases the two hemispheres were not distinctly separated, and in some extreme cases they merely formed (in transverse section) a compressed tubular structure (Plate, fig. B). The wax-plate reconstructions show, however, that in these cases there is really a vesicle folded and compressed in all directions. A characteristic feature of the hemisphere overgrowth was the presence of a more or less distinct protrusion or pouch of the lateral wall of the hemisphere (Plate, figs. A, C), which was either unilateral or bilateral. There was always a marked asymmetry in form between the two halves, and in some cases asymmetry in size.

There was usually a strong folding of the mesencephalic roof (Plate, figs. A, B). The separation of the tectum opticum vesicles was sometimes incomplete. In some embryos there was an encephaloschisis with strongly everted lateral parts, bending ventralwards. As with the hemispheres, there was always asymmetry with regard to size and shape of the two brain halves in the mesencephalon. In the material operated upon by Hugosson, parts of neuromere *c* were also removed—i.e. the future mesencephalic anlage; hence these embryos showed only a partial development of the tectum opticum. In these parts the overgrowth phenomenon was strongly marked.

The degree of overgrowth in the mesencephalic roof was highly variable and was sometimes practically absent. There is no relation between the degree of the overgrowth in the hemispheres and in the tectum opticum vesicles.

In some cases the epiphysis also developed signs of overgrowth. The diencephalic roof, which normally forms a single dome, may be divided into two ridges, one of them carrying the epiphyseal rudiment, or it may be completely transformed into an overgrowth.

In some cases, clusters of cells grew into the ventricular lumen in locations where overgrowth was present (Plate, fig. B). These clusters resemble the cell rosettes, described in the telencephalic chorioid plexus by Kappers (1957; his fig. 9). In fig. 18 in that paper, Kappers also showed tumour-like structures resembling the formations observed in the present writer's material. There are numerous mitoses in his structures.

There is a left-sided microphthalmia in three embryos and overgrowth was found in the optic stalks and vesicles of three others.

In no single embryo belonging to the present material have signs of overgrowth been observed in the rhombencephalon or in other parts of the brain.

In the five embryos in which no signs of overgrowth could be found, in spite of the fact that neuromere *d* had been extirpated, the sections and the reconstructions show that the notochordal tip had not been removed.

Control operations

After extirpations of neuromeres situated caudad and rostrad to neuromere *d*, no signs of overgrowth could be seen except in three embryos in which neuromere *e* had been extirpated. The sections show, however, that neuromere *d* had also been damaged and probably, too, the notochord underlying neuromere *d*.

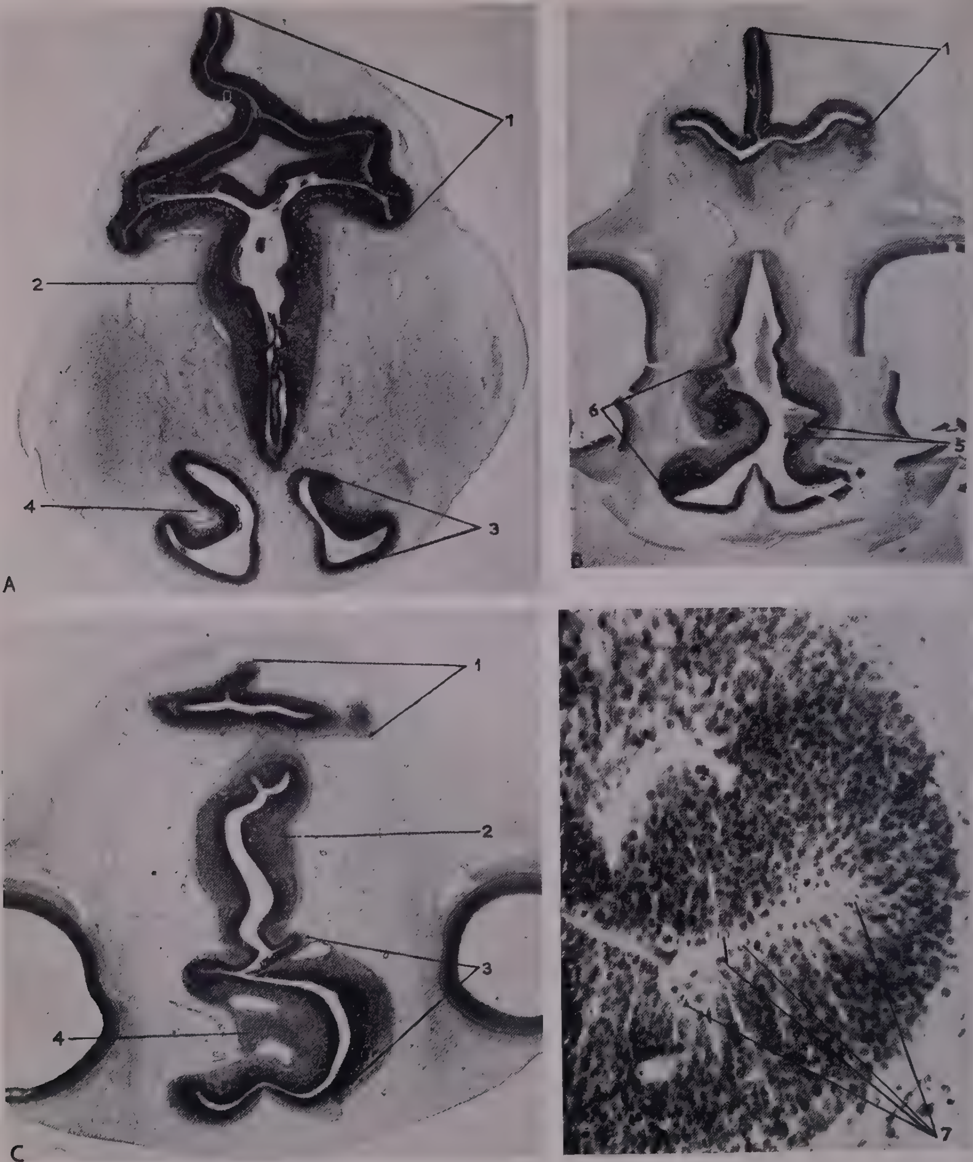
DISCUSSION

In the introduction to the present paper it is pointed out that Sjodin obtained formations in chick embryos resembling the overgrowth phenomenon after either hypoxia or chemical influence. Sjodin regarded the phenomenon as a post-mortem change. He supposed that the same was true for the malformations described by Patten.

The overgrowth described in the present paper occurs in embryos which are alive and apparently otherwise normal and is also of a different nature. In fig. D of the Plate part of the mesencephalic overgrowth in embryo 9 (see Plate, fig. A) is shown at high magnification. It may be compared with fig. 6 A and B, p. 600, in Sjodin's paper. In the present material no pyknotic nuclei or autolysed cells can be seen, but numerous mitoses, showing that the tissue is strongly proliferating. It is possible that the spontaneous overgrowth phenomena described by Patten and others are of a similar nature. It cannot be shown that the teratogenetic factor, operating in spontaneous overgrowth, acts upon the same region as that damaged in the present operations, although this possibility is not unlikely. In some of Sjodin's experiments damage to this region may also have occurred. Whether the influence of the notochordal tip is essentially inductive in character has yet to be shown experimentally; but it is striking that Eyal-Giladi (1958) has demonstrated a specific action by the notochordal tip in the formation of the orohypophysis.

SUMMARY

1. A pathologic phenomenon consisting of complex folding of the brain-wall has been obtained in living chick embryos after operations upon the rhombencephalon in early stages. The operation consisted of the extirpation of the tip of the notochord and the overlying neuromere. Its results mainly appear in the hemispheres and tectum opticum region, but sometimes also extend to other



H. BERGQUIST

evaginated brain parts, viz. the optic evaginations, the epiphyseal region, and possibly also the caudal parts of the hypothalamus.

2. The pathologic phenomenon is identical with certain abnormal foldings, described in human embryonic brains by Patten (1952) and called by him 'overgrowth'. It may be combined with encephaloschisis in the mesencephalic roof.

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EXPLANATION OF PLATE

KEY: 1, tectum opticum in overgrowth; 2, thalamus; 3, left hemisphere in overgrowth; 4, pouch of lateral wall of hemisphere; 5, cell clusters; 6, right hemisphere in overgrowth; 7, cells in mitosis.

FIG. A. Transverse section of embryo 9, cutting the caudal parts of the hemispheres, the thalamus, and the mesencephalon. There is a distinct overgrowth in the hemispheres and mesencephalon. See also fig. D. ×22.

FIG. B. Transverse section of embryo 9, showing the cell clusters extending from the neural epithelium into the ventricular lumen. ×22.

FIG. C. Transverse section of embryo 35, showing overgrowth in the hemispheres. ×22.

FIG. D. Detail of the mesencephalic overgrowth in embryo 9. The section shown here lies one section in front of that shown in fig. A. ×255.

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Studies in Embryonic and Larval Development in Amphibia

II. The Spinal Motor-Root

by ARTHUR HUGHES[1]

From the Anatomy School, Cambridge, and the Zoology Department of the University College of the West Indies

WITH TWO PLATES

INTRODUCTION

In 1913 G. E. Coghill initiated a series of papers on the neuro-embryology of the Urodele *Ambystoma* with a description of the earliest stages of the motor system of the trunk (Coghill, 1913). His main conclusion is stated early in the paper in these words:

> The neurones . . . which establish the earliest contact with the cells of the myotome are found in *Amblystoma* to be at the same time the neurones of the motor tract in the central nervous system. The primary ventral root fibre is a collateral of the tract cell. (Coghill, 1913, p. 121.)

Thirteen years later, among a group of other papers on the developing nervous system of *Ambystoma*, he returned to this theme, and in a series of examples described the form of the first nerve process within the basal plate of the cord. In all of these, which belong to early phases of motility of the embryo, a primary motor-cell is shown with two branches; one leaves the cord to meet the inner surface of a myotome, the other forms an element of the longitudinal motor-tract of the white matter. The precise form of the branching varies from one example to another. Thus, again to quote Coghill:

> In longitudinal sections properly directed . . . the root fibres are commonly seen turning out of the tract from cephalad . . . however, it is only in the third, fourth, and fifth spinal segments . . . that root fibres have been seen going directly out of the spinal cord from nerve cells at the same levels. (Id., 1926, pp. 97, 98.)

According to this author therefore, the longitudinal motor-tract of the cord originates at the same time as the ventral roots, none of which arise independently of fibres of the white matter. Only from a minority of primary motor-cells, however, do branches emerge from the cord:

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> It is obvious that . . . from the small number of motor-root fibres as compared with the large number of primary motor cells . . . that by far the larger number of these neurones function as tract neurones exclusively. (Ibid., p. 108.)

Twelve years later Youngstrom (1938) published figures of the earliest axonal processes of the cord, both in *Ambystoma* and in *Rana*, which bifurcate as Coghill described. Again, a longitudinal element of the white matter and a ventral-root fibre arise simultaneously. As with Coghill, these first branched axonic processes were seen in the first stages of motile behaviour, when the embryo responds only to sudden pressure, or occasionally makes spontaneous movements.

Two years before the appearance of Youngstrom's paper, however, Szepsenwol (1936) denied that spinal motor-roots were present in *Ambystoma* as early as the stages in which Coghill described them. According to Szepsenwol, the third spinal motor-root has not yet grown beyond the limits of the cord in an 8-mm. larva, the behaviour of which has already entered the early swimming stage. Szepsenwol, however, is correct in claiming that a myogenic phase of behaviour must precede the neurogenic pattern, though the purely negative evidence that his particular method of silver impregnation fails to reveal ventral-root fibres before the animal can swim is inadequate. Boell & Shen (1950) have since shown that throughout the early flexure and coil phases of development no cholinesterase is detectable within the cord, and that the enzyme first appears just before swimming behaviour begins, and when the embryo first responds neurogenically to tactile stimuli.

Szepsenwol and Coghill, however, are in tacit agreement on the early origin of the motor-tract in *Ambystoma*. On this point there is no evidence at present to the contrary; indeed, if Szepsenwol (1936) were right, ventral roots in *Ambystoma* lag far behind the development of the longitudinal tract, for his transverse section of an 8-mm. larva, where the third spinal root is claimed not yet to have left the cord, shows an obvious layer of white matter, about 30 μ in thickness on the ventro-lateral surface of the cord.

The present paper is concerned not only with the first origins of the amphibian ventral root, but also with its interrelationships with the longitudinal tract at later stages. The primary aim was to examine what differences there are in these respects between *Eleutherodactylus*, which has a wholly embryonic development, and other Anura with a normal larval phase in their life-history. In an attempt to define the characteristic features of the developing amphibian ventral root, however, some observations on higher vertebrates have been included.

MATERIALS AND METHODS

The observations described in this paper are based on a collection of silvered series of sections of a number of vertebrate embryos which has gradually been acquired in recent years. The *Xenopus* material is the same as that used in other recent neuro-embryological studies (Hughes, 1957; Hughes & Tschumi, 1958;

Lewis & Hughes, 1957) together with others yet to be published. The embryos of *Eleutherodactylus*, *Bufo*, and *Sphaerodactylus* were collected on a recent visit to Jamaica.

In the early years of building up this collection, the method of silver impregnation on the slide of Holmes (1947) was used, and satisfactory results for the most part were obtained with the chick, though perhaps less often with embryos and larvae of *Xenopus*. Recently, however, a satisfactory silver proteinate now being available (Établissements Roque, Paris), the Bodian technique has been used exclusively with more uniform results than is possible by Holmes's procedure.

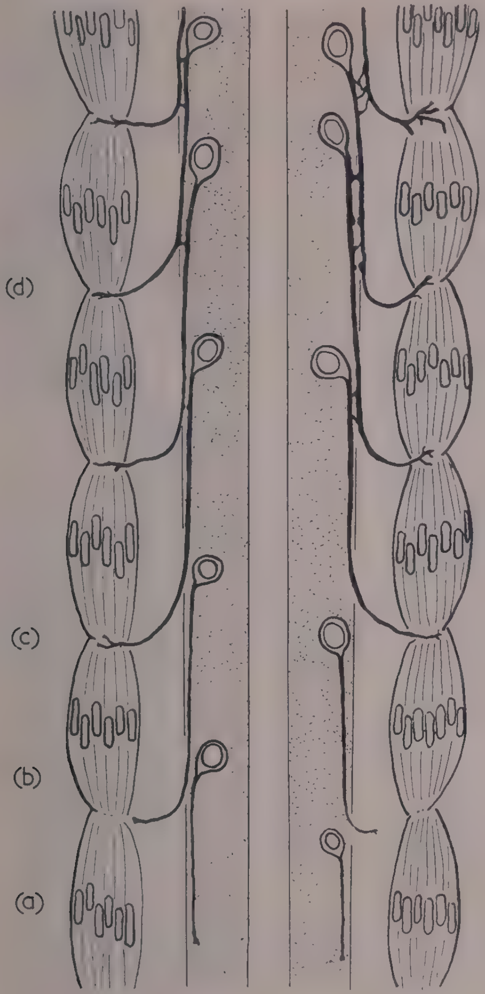
The stages of embryos and larvae of *Xenopus laevis* are given as those corresponding most closely to the descriptions of Nieuwkoop & Faber (1956). In early larvae, however, we have observed some discrepancies between our batches and those of the Dutch authors. Ours hatch at N. and F. 30, instead of N. and F. 34, and are about half a millimetre longer at this period of development. Differences in temperature may well be responsible for these variations.

XENOPUS LAEVIS

In *Xenopus* it is probable that the earliest movements of the embryo belong to a myogenic phase. Nieuwkoop & Faber (1956) state that at their stage 24 there are initial motor reactions to external stimulation; spontaneous movements begin at stage 26. In our material at stage 25 I am unable to detect any processes from cells of the neuroepithelium either within the cord or beyond it. Moreover, the esterase reaction of Lewis (Lewis & Hughes, 1957; Lewis, 1958) is first detectable in the myocommata only at stage 29, which on the time-scale of the Dutch authors is reached some 8 hours later than the earliest movements are apparent. The presence of esterases at first is confined to the anterior two-thirds of the trunk; within these same limits we then see a terminal myocommatal plexus of motor fibres in silvered sections.

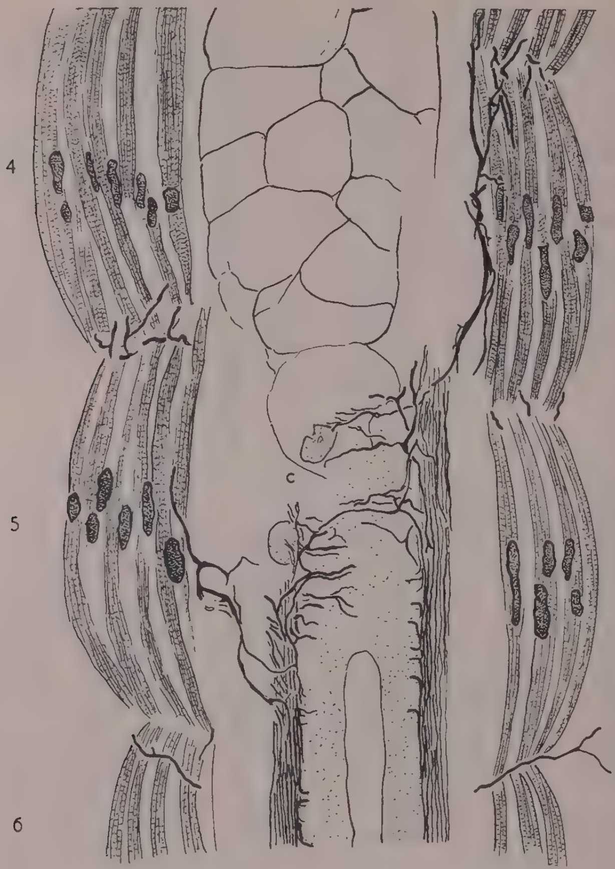
Nieuwkoop & Faber (1956) state that white matter is present in the anterior half of the spinal cord at stage 28, and has extended over 70 per cent. of its length by stage 31. The ventral roots become functional during these stages and the ventro-lateral surface of the cord is soon covered with a layer of white matter up to six fibres in depth, and about 8 μ wide (Plate 1, fig. A). Thus in *Xenopus* the development of the longitudinal motor-tract is not preceded by the formation of ventral roots.

In horizontal and coronal sections of embryos and young larvae at this period of development, details of the formation of ventral roots and of the longitudinal motor-tract can be observed at the hinder end of the cord, and the successive stages in the process can be followed by tracing forwards from levels where no fibres are yet visible (Text-fig. 1). First, from a future primary motor-cell an axonal fibre grows caudalwards along the surface of the neural tube. In this way arise short unconnected lengths of the longitudinal tract. Next, the tips of these fibres turn outwards at inter-myotomic levels, and grow across the myocommatal planes. By this stage, the proximal longitudinal parts of these axons overlap, and the white matter then consists in places of more than one layer of fibres. Next, a cross-anastomosis develops between adjacent axons, and the whole pattern is



TEXT-FIG. 1. Diagram of the formation of ventral roots in embryos of *Xenopus*, as seen in horizontal sections through the cord and myotomes. Successive stages seen in caudo-cranial sequence. (*a*) Growth of longitudinal fibre. (*b*) Tip of fibre turns outwards opposite myocomma, and (*c*) forms intermyocommatal plexus. (*d*) Cross-anastomoses develop between adjacent axons.

finally completed by the development of commissural processes across the mid-ventral line of the cord. A ventral root in *Xenopus* is thus the terminal portion of the axon of a primary motor-cell; from the first the perikaryon is at a different transverse level from that of the myocommatal termination of the axon (Plate 1, fig. C). The earliest longitudinal processes in the cord have a weak affinity for silver, but are sufficiently distinct to establish the fact that in the first place they are quite unbranched. Thus the sequence of development of the ventral roots of



TEXT-FIG. 2. Drawing of near-horizontal section through larva of *Xenopus* at stage N. and F. 43. The section traverses trunk myotomes 4 and 5, and descends cranially into the notochord. A forwardly directed ventral root, originating from the deeper layers of the longitudinal motor-tract, is shown on each side. That on the right continues to its intermyotomal plexus. Between trunk myotomes 5 and 6 are seen afferent fibres of Rohon–Beard cells. *c*, fibres of the ventral commissure. ×400.

Xenopus is not the same as that which Coghill described in *Ambystoma*.[1] The end result, however, is similar, although in *Xenopus* the elements of the longitudinal tract are recognizable earlier than are the ventral roots. In this Anuran an essential feature of the arrangement is the relative longitudinal displacement of cell-body and axon termination.

This displacement, however, is not uniform throughout the length of the cord. In the tail the motor roots arise from the surface of the cord at so small an angle to its long axis that the point at which the individual fibre is first free of the outer layers of the white matter is several segments in advance of the myotome which it innervates. At limb-bud stages of the larva, ventral roots pointing tailwards are also found in the hinder region of the trunk, at the levels from which the hind limb is innervated. In the upper part of the cord, however, the motor roots at first point in the opposite direction, and run forwards to the myocommata (Plate 1, fig. B) (Text-fig. 2). At stage N. and F. 34, forwardly directed ventral roots innervate the first eight post-otic myotomes; of these the first four belong to the head, and will later disappear together with the first of the trunk series (Nieuwkoop & Faber, 1956, p. 108). The longitudinal motor-tract which covers the ventro-lateral surface of all but the posterior fifth of the cord consists of many layers of fibres. Between cross-anastomoses they run parallel with each other only for short distances, and the whole white matter in longitudinal section has the form of a meshwork with elongated interstices. To this meshwork axon roots descend in transverse planes from their cell-bodies. Some branches cross the midline to end in the longitudinal tract of the other side without the interposition of any floor-plate cells such as Coghill (1924) describes in *Ambystoma*.

The abrupt change in the angle at which the ventral roots first arise invites the question whether this has any bearing on the neuro-muscular control of swimming movements in *Xenopus*. Coghill (1926) transected the cord of *Ambystoma* at various transverse planes, and showed thereby that a centre for the co-ordination of swimming was located between the otic capsule and the forelimb. Accordingly, the same experiment was performed on tadpoles of *Xenopus* at stages N. and F. 31–34, soon after hatching, when the larva is already swimming spontaneously. The central nervous system was transected at three levels: in front of the otic capsule, immediately behind it, and several somites behind it (Text-fig. 3). In the first instance, the larva immediately began to swim vigorously; in the second, movement of the trunk was reduced to a spasmodic twitching; and in the third, all motion ceased. These results suggest that in *Xenopus* also, muscular activity in the trunk is governed by a centre just behind the otic capsule. At this stage this level coincides with that of the fourth head somite (Nieuwkoop & Faber, 1956, p. 35) and is within the series of forwardly directed ventral roots. (The Mauthner cells are at this stage present at a level immediately behind the otic capsule, though their long axons are not yet distinct.) The same experiment was repeated on the following day, when the larvae had reached stage 39, with the same general result except that any interference with these later tadpoles rendered them immobile for some minutes.

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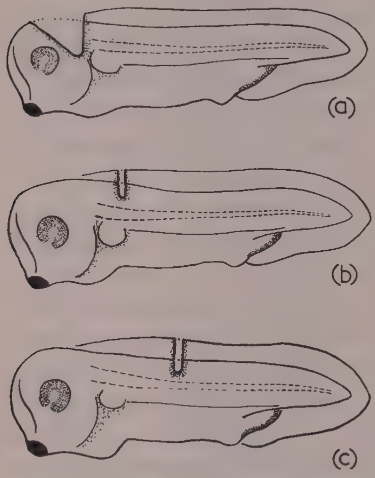
[1] Although Coghill's (1929; see Fig. 11) diagrams of the motor innervation in the larva of *Ambystoma* show fibres ending in the middle of a somite, yet in his earlier paper (1926, pp. 101, 102) myocommatal endings to some branches of the ventral roots are described.

A point of some importance concerning the disposition of the spinal roots in the early larva of *Xenopus* is that whereas the ventral roots are inclined at acute angles to the cord, pointing either forwards or backwards, the sensory nerves do not share in this arrangement. The afferent fibres of the Rohon–Beard and Fritsch cells (Hughes, 1957), which run from myocommata to the dorsal surface of the cord, are all nearly at right angles to the longitudinal axis throughout the length of the neural tube. This difference between sensory and motor fibres at these stages suggests that the arrangement of the latter is not achieved by any general process of differential growth between somites and spinal cord.



TEXT-FIG. 3. Effect of transection of brain and cord on swimming movements of *Xenopus* larvae between stages N. and F. 31–33. (*a*) Removal of mid- and hind-brain in front of otic capsule results in immediate and continued swimming. (*b*) Transection immediately behind the level of otic capsule reduces movement of trunk to spasmodic twitching. (*c*) Transection well behind otic capsule renders the body behind the cut immobile, and unresponsive to stimulation.

As larval life progresses and the motor column of white matter in the cord increases still farther in thickness, it becomes possible to recognize a second distinction between the ventral roots in the upper and lower regions of the cord. A group of the latter peels off, so to speak, and forms a ventral root, but in the upper region of the cord, where the ventral roots at first ran forwards, some of their constituent neurites can now be traced inwards through the white matter to an origin from fibres on its inner surface (Text-fig. 2). Such deep ventral-root fibres, as they may conveniently be termed, cross the longitudinal tract at various angles, but their outward course, although often irregular, does not involve any anastomosis with surrounding fibres. The presence of deep ventral-root fibres in the upper part of the cord can be recognized as early as stage N. and F. 39.

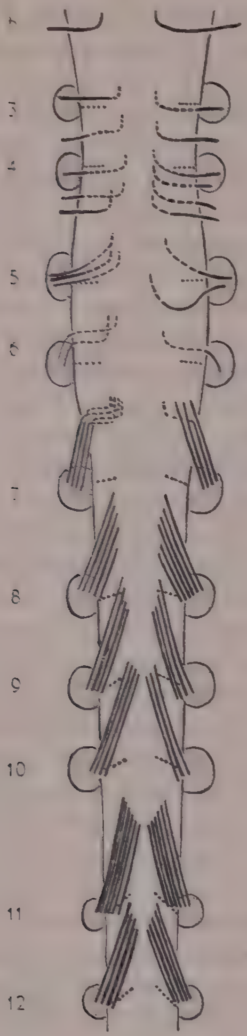
In the later stages, the angle between the deep ventral-root fibres of the trunk and the long axis tends to approach a right angle, both within the white matter and for the first part of their course outside the cord. Their essential similarity in arrangement to the ventral roots of earlier stages is however clear, thanks to the later growth in diameter of some of the constituent fibres. At stage N. and F. 52, when the hind limb-bud is 2 mm. long and the ventral horns of the cord are in the neuroblast stage of development (Hughes & Tschumi, 1958), there is a great range in calibre of fibres within the white matter. The maximum diameter of the two Mauthner axons is about 15 μ; grouped around them are a number of large descending fibres up to 6 μ in diameter.

A few elements of the medial tract of large fibres originate in the hindbrain. As the tract descends, their number on each side increases from three to about twelve on each side at the level of spinal ganglion 5. A proportion of the ventral commissural fibres of the trunk are also conspicuously large. They become much rarer as one proceeds backwards into the tail region, and are soon lost altogether beyond the first few tail segments. In Text-fig. 4 is shown the emergence as ventral roots of those fibres which are 3 μ or more in diameter. Their number within the ventral roots increases from one at upper levels of the cord to six or more in the tail. Here a transverse section of the cord at this stage has a striking appearance (Plate 1, fig. E). A medio-ventral zone of the white matter, together with a group of ventral roots, are mainly composed of large fibres; their diameter approaches that of the nuclei in the mantle layer of the grey matter of the cord. It is likely that these giant axons, capable of conducting impulses much more rapidly than are smaller fibres, are related to the peculiar flickering motion of the tail-tip in the later larval stages of *Xenopus*. This movement is constantly maintained throughout the long periods when in undisturbed larvae the swimming movements of the trunk and anterior tail region are at rest.

The same difference between the motor roots at upper and lower levels in the cord is still recognizable. Ventral roots in the tail now originate near the median ventral sulcus in the cord by the gradual detachment of a group of fibres; the large ones carry with them a number of finer elements, a micron or less in diameter. This type of origin of ventral roots extends forwards through the levels of supply of the hind limb, namely, those of dorsal root ganglia 8, 9, and 10.

Throughout the remaining upper portion of the cord the large fibres of the ventral roots are reduced in number to one or two to each root. Each is given off from a descending tract surrounding the Mauthner axon and turns through a right angle to emerge from the white matter on the lower surface of the cord (Plate 1, fig. D); such fibres correspond to the deep ventral-root fibres already described for earlier stages of development, though due to the loss of the upper myotomes and the concomitant changes in relative position of axial and peripheral structures, the anterior spinal motor-roots at stage N. and F. 52 no longer run forwards to their myotomes.

The ventral roots at the level of dorsal root ganglion 7 contain four large fibres each. On one side all originate deeply, while on the other, only one is of this kind. At this level there is thus a transition from roots of the anterior trunk to those of the posterior region of the cord. The hypoglossal nerve, which is the ventral root corresponding to the lost spinal ganglion 2, unlike other ventral roots at upper levels of the cord, originates entirely from superficial fibres (Text-fig. 4).



Text-fig. 4. Reconstruction of ventral aspect of spinal cord of *Xenopus* larva at stage N. and F. 52, to level of spinal ganglion 12, showing origin of large motor-root fibres. From the level of spinal ganglion 8 tailwards, these arise from the surface layer of the white matter. In the trunk, the inner medial layers of the longitudinal motor-tract give off large fibres which run through the white matter (dotted lines), and emerge as large ventral-root fibres. The hypoglossal nerve (*h*) originates superficially. The course of the corresponding dorsal roots are shown by round dots.

Despite the differences in arrangement at upper and lower levels in the cord, in late larval stages it can clearly be seen that, thanks to their conspicuous calibre, some fibres of all ventral roots are derived from elements of the longitudinal tract of the cord.

In Text-fig. 4, together with the ventral roots of the cord, the corresponding dorsal roots are also shown. One difference in arrangement of the latter between those of trunk and tail is that while the former run dorsally over the surface of the cord entering at a point near the apex, incoming fibres from the large neurones of the tail ganglia (Hughes & Tschumi, 1958) enter the white matter ventro-laterally, inside which they ascend towards the alar plate. Furthermore, the anterior members of the series of dorsal roots are at right angles to the cord; caudalwards from spinal ganglion 7, however, they deviate increasingly from this direction, though always to a lesser extent than do the corresponding ventral roots.

Fibres from the dorsal root ganglia first enter the cord at stage N. and F. 46, when the whole series run uniformly at 90 degrees to the longitudinal axis. It is probable that between stages 46 and 52, which on the time-scale of Nieuwkoop & Faber represents an interval of 15 days, the deviation from the original right-angled condition of the dorsal roots is derived by a differential growth in the axial direction whereby the spinal cord elongates less rapidly than the surrounding myotomes. Such a relative displacement would affect both dorsal and ventral roots, the original inclination of the latter of which would be still further exaggerated. This secondary shift, however, must be clearly distinguished from the original inclination of the ventral roots which is present from the first, and which appears to be a specifically larval amphibian feature.

BUFO MARINUS

A series of tadpoles of this species was collected from a lily-pond in the Hope Botanical Gardens, Mona St. Andrew, Jamaica. Some larvae were cut in the horizontal plane and silvered by Bodian's method. The following description is based on one such section series, of a larva 9·3 mm. long, in which the hind limbs were 1·6 mm. in length, with their digits already well marked. Inside the spinal cord melanin granules are very common within the cytoplasm of many cells; the pigment is concentrated mainly at the surface of the grey matter, within differentiated neurones, and also in a narrow mid-ventral band of the cord. The same distribution is seen at earlier stages and in unsilvered sections.

One difference between this larva and that of *Xenopus* at a corresponding stage is the near-uniformity in calibre of nearly all fibres within the cord. Even the axons of the Mauthner cells are only about 2 μ in diameter, and their cell-bodies in the medulla are little larger than those of the surrounding neurones and can only be identified with certainty by the decussation of their axons.

The arrangement of the ventral roots in the tail of this larva of *B. marinus* is very similar to that in *Xenopus*. They arise from the surface layer of the white matter, and are given off at very small angles to the axis of the cord. Again, in the trunk the arrangement recalls that of the same region of the cord in *Xenopus*, in that the motor roots arise from deep ventral-root fibres. These roots emerge from the cord at various angles. Each can be traced back into the cord, and then in successive sections through the white matter as a distinct bundle running forwards, inwards, and up towards the central canal (Plate 2, fig. A). As the ventral sulcus is approached, and close to the ventral commissure, each bundle turns sharply forwards to merge completely with the inner part of the longitudinal motor-tract.

The motor roots of the lumbar nerves consist of two types of fibres. Relatively coarse bundles arise from the surface of the white matter, while elements of smaller calibre can be traced through the outer zones of the longitudinal tract to their origin from its inner layers. This group of fibres closely resemble those of the more anterior ventral roots of the trunk.

Although the first origins of the ventral-root fibres in *Bufo* have not been traced, the study of this single stage is sufficient to demonstrate that their general arrangement is similar to that in *Xenopus* both in their relationship to the longitudinal tract and in the angles at which they arise.

ELEUTHERODACTYLUS

In a previous paper (Hughes, 1958) the general features of the embryology of *E. ricordii* have been described together with some points in the development of the spinal cord and dorsal-root ganglia, mainly with regard to the embryonic development of this amphibian and the particularly rapid ontogeny of this species. The absence of any larval phase in the life-history affects the whole course of development; in the central nervous system the Mauthner cells of the medulla are suppressed, the Rohon–Beard system of primary sensory neurones does not precede the development of the dorsal-root ganglia, and the white matter of the cord is much less conspicuous than in larval Amphibia.

The material collected in Jamaica and described in the previous paper served also for observations on the development of the ventral roots. The calibre of the fibres throughout the central nervous system is extremely fine, and the peripheral nerves are so abundantly supplied with Schwann cells that the fibres which these cells accompany are almost obscured.

In *E. ricordii*, at a stage 10 days from hatching (which, following the usage of Lynn (1942), is designated as – 10 days), the ventral surface of the spinal cord is covered by a thin layer of longitudinal fibres which, in the first place have grown downwards from the medulla. (Such fibres are present in the embryos of all vertebrates, but are obscured in larval Amphibia by the precocious development of longitudinal fibres of spinal neurones.) Within the thick neuro-epithelium of the cord there can be observed early stages in the outgrowth of nerve-processes, none of which could yet be traced with certainty to the surface.

At this stage the living embryo exhibited an occasional wriggling motion, both spontaneously and also after slight mechanical shock. This movement apparently corresponded with the 'early flexure' phase of behaviour in *Ambystoma*, and in *Eleutherodactylus* also it may be entirely myogenic in origin. On the succeeding days, however, it was never seen to build up into a co-ordinated swimming movement. The embryonic behaviour of *Eleutherodactylus* demands further study, but the conclusion that the usual swimming movement of larval Amphibia is absent is supported both by the fact that the embryo is curled round a relatively large globe of yolky endoderm, and also by the consideration that within the egg envelope there is no unoccupied space to permit of swimming activity.

At –9 days neuroblasts within the trunk region of the cord have developed two systems of axons. A single layer of decussating fibres cross the midline, and, secondly, ventral roots have emerged from the surface of the cord. These traverse a layer of white matter still only a few fibres in thickness. The course of the ventral-root fibres is nearly at right angles to the axis of the cord. They run between the grey matter and the outer surface of the cord entirely independently of the longitudinal fibre tract (Plate 2, fig. B). Study with an immersion lens of sections cut in the plane of the ventral roots clearly shows that longitudinal and transverse fibres cross at different levels.

At –9 days no longitudinal fibres or ventral roots are yet to be seen within the tail. In *Eleutherodactylus* the tail develops in the first place sharply twisted through a right angle at its union with the trunk, so that the notochord and spinal cord lie side by side with the dorsal and ventral fins spread outwards in a horizontal plane. A few longitudinal fibres reach the tail spinal cord at –8 days, but the white matter of this region remains extremely sparse during the whole of development. Nevertheless, the arrangement of ventral roots and longitudinal fibre tracts can be shown to be similar in the tail of *Eleutherodactylus* to that in larval amphibia. The ventral roots consist each of a single axon. A fibre of the descending longitudinal tract leaves the cord at an acute angle, and makes its way laterally and caudally towards the slender myotomes of the tail (Plate 2, fig. C), where it can be traced as far as an inter-myotomic septum.

In *Eleutherodactylus* the lumbar ventral roots arise wholly from deep fibres. Their course can readily be traced in embryos at –6 days. The ventral horns are covered only by a thin layer of white matter through which, at various angles, the axons of motor-cells run directly to the surface to emerge as the ventral roots of spinal nerves 8, 9, and 10.

Thus, whereas both *Eleutherodactylus* and larval Anura exhibit the same pattern of ventral roots in the tail, their respective arrangements in the trunk region are wholly dissimilar, for in *Eleutherodactylus* the origin of the longitudinal motor-tract and of the ventral roots is entirely distinct.

OBSERVATIONS ON SAUROPSIDA

In order to compare the ventral roots of developing Amphibia with those of higher vertebrates, and so to define more closely the specific features of each, embryonic stages of two Sauropsida were examined. For this purpose silvered series of embryos of a lizard and the chick were available.

The small Geckonid lizard *Sphaerodactylus* is common in Jamaica. Several species of the animal were described by Hans Sloane (1725) as the 'Wood Slave', by P. H. Gosse (1851) as the 'Palette-tip', and are known to the modern Jamaican as 'polly lizards'. A Cuban species is only 17 mm. long when full grown and must be the smallest of all Tetrapods.

Eggs of *Sphaerodactylus* are about 5 mm. long: a number were collected in various damp places in Jamaica. The youngest embryo measured 4·5 mm. from midbrain to the curved dorsal surface of the trunk. Each limb-bud ends in a broad paddle, and the general stage of development is that of a 5-day chick. The oldest embryo was much more mature. The digits already terminate in the rounded expansions from which the name of the genus is derived; the head is unmistakably Saurian. Over the surface of the body, folds of skin indicate the future scales; the dermis is heavily loaded with melanophores. Within the skeleton ossification has begun only within the centra of the vertebrae, although some cartilage of the limb-skeleton has hypertrophied.

In the spinal cord of the earlier of these two embryos the ventral roots are all of a uniform arrangement throughout the series. Each is formed by the convergence within the white matter of a series of fibres which approach their point of emergence from the cord in various directions. Fibres in the middle of each group run directly from grey matter to the surface; those on either side take a more oblique course. In succeeding sections, bundles of these ventral roots can be traced through the white matter to their origins from neuroblasts of the basal plate. No anastomosis between ventral roots and elements of the longitudinal tract can be detected.

In the later embryo of *Sphaerodactylus* the arrangement of the spinal motor-roots in the anterior region of the trunk is similar to that at the earlier stage, except that the whole root is now inclined caudally. There is the same convergence of fibres upon the point of emergence of the root, but they are now no longer arranged symmetrically on either side of a transverse plane. At the cranial border of the root, the constituent fibres run at an angle of 45° or even less through the white matter. Even here, however, it is clear that their course is independent of the longitudinal tract (Plate 2, fig. D).

The inclination of the ventral root is shared equally by the dorsal root at the same level. In both series the angles which they make with the cord decrease progressively caudalwards until at the tip of the tail no clear distinction can be made between white matter and spinal nerves at their origin. Comparison with the earlier embryo shows that this condition is a secondary one; it must be caused by changes in relative growth which affect both dorsal and ventral roots equally, and which are continued into post-embryonic life. In the tail of adult individuals of *Sphaerodactylus* the proximal portions of both dorsal and ventral roots which lie within the neural canal of the vertebral column are greatly elongated and are given off at very small angles to the cord.

Finally, the ventral roots were studied in the chick embryo, where in common with other higher Amniotes, the motor and commissural neuroblasts in the cord differentiate entirely independent of the longitudinal tract of the white matter as is shown in published figures of the development of the cord (Ramon y Cajal, 1909; Tello, 1922; Windle & Orr, 1934; Barron, 1946; Hughes, 1955). Section series were studied at 8 and 9 days of incubation. Where the plane of section is parallel to the spinal cord it can clearly be seen that each ventral root derives its fibres from a much shorter segment of the cord than in *Sphaerodactylus*, for their course in the chick is unvaryingly parallel, and deviates from transverse planes by only a few degrees. This is true not only for the ventral roots at limb levels, but also for the last spinal nerve of all, the 38th of the series, where the uniform arrangement is maintained. Up to this stage, the chick cord remains without any secondary displacement of the spinal roots.

DISCUSSION

The second of Coghill's papers on the ventral roots of *Ambystoma* ends with a comparative section in which he maintains that their 'mode of origin . . . is not an anomaly among vertebrates' and cites several instances where earlier authors had described early spinal motor-roots from which longitudinal fibres are given off as branches of the same axon. His examples include *Amphioxus*, the ammocoete larva of the lamprey and both cartilaginous and bony fish. However, for at least two of these, the detailed and scholarly researches of Whiting have since shown that ventral roots precede the appearance of longitudinal fibres in the cord. Thus in the pro-ammocoete larva of *Lampetra* (Whiting, 1955), when trunk movements are already under nervous control, the ventral-root fibres of the primary motor-cells run straight outwards from the cord to the myotomes. Longitudinal branches of these axons do not appear until the ammocoete stage is reached (Whiting, 1948). Again, in the trout embryo, ventral roots make contact with the myotomes early in the myogenic stage: 'the somatic motor neurone developing in the order peripheral axon, dendrites, longitudinal collateral of the axon in the ventral column' (Whiting, 1955, p. 96). Study of this important review suggests that the early origin of the longitudinal motor-tract may in fact be a characteristic feature of the neuro-embryology of larval Amphibia, and one in which a distinction may be drawn between them and lower chordates.

In the present paper, evidence from the development of the motor system in *Xenopus* supports this thesis, and suggests that its acceptance does not necessarily commit one to a belief in Coghill's description of the first origin of the motor system in *Ambystoma*. It is not impossible that the earliest stages of the formation of the motor system may differ in detail among various Amphibia. If the pattern of development which has here been described for *Xenopus* is widespread among Amphibia, a second general feature of their neuro-embryology would be the longitudinal displacement of cell-body and motor ending. This is a primary and necessary concomitant of the manner in which motor roots are formed in *Xenopus*, though the present observations on the ventral roots in embryos of *Sphaerodactylus*, incomplete as they are, suggest that there may be no absolute distinction between Amphibia and reptiles in this respect. Lizards such as *Sphaerodactylus* walk by combining the original swimming movements of the vertebrate body with the action of the limbs. It may be that the co-ordination of swimming movement requires some degree of obliquity of the ventral-root fibres within the white matter of the cord, whereby each motor root receives fibres from a relatively wide longitudinal segment of the cord.

In this respect *Eleutherodactylus*, in which swimming movements are absent in both embryo and adult, shows a more advanced and specialized type of motor innervation of the trunk myotomes than does *Sphaerodactylus*. We need, however, much more information on the neuro-embryology of a wide range of vertebrates before firm conclusions on this and other questions can be drawn. It would be of great interest to study the motor pattern in the early stages of other Amphibia which share with *Eleutherodactylus* some prolongation of the embryonic phase of development.

Meanwhile, the thesis implicit in the present account of the development of the motor system in *Xenopus* and *Eleutherodactylus*, i.e. that the likelihood of cross-anastomosis between growing nerve fibres depends on the angle at which they approach one another, receives some support from observations on the growth of embryonic neurites in tissue cultures which have been described incidentally in a previous paper (Hughes, 1953). It was there observed that if two fibres meet each other at right angles, contact results in a mutual inhibition of growth, and one can only cross the path of the other by avoidance at a different level. Thus where, as in the trunk of the *Eleutherodactylus* embryo, ventral-root and longitudinal fibres approach each other at right angles, they necessarily remain independent. Where, however, fibres grow towards each other at acute angles, they tend to run parallel and to form cross-connexions which may persist for varying periods. In the late embryo of *Xenopus*, the relationships of the ventral roots and the longitudinal tract with their irregular and plexiform anastomoses are reminiscent of this type of interaction. To define, however, the limits of individual neurones within such an arrangement is at present as difficult in a living tissue-culture as in a silvered section of an embryo.

SUMMARY

1. The development of the spinal motor-roots is described in the Anurans *Xenopus* and *Eleutherodactylus*, together with observations on the embryonic roots of *Bufo*, the lizard *Sphaerodactylus*, and the chick.

2. From a primary motor-cell in the embryo of *Xenopus* an axon process grows along the surface of the cord and finally turns outwards to traverse a myocommatal plane, thereby forming a ventral root. As in other larval Amphibia, the longitudinal motor-tract and the ventral roots originate together, though not necessarily by identical methods in all.

3. In the recently hatched larva, movement is inhibited by transection of the central nervous system behind the otocyst. At these levels there must be a centre for the co-ordination of swimming movements.

4. In mid-larval stages the ventral roots of *Xenopus* and *Bufo* arise from the longitudinal motor-tract of the cord; cell-body and myocommatal terminal of the axon are longitudinally displaced, though the precise arrangement varies at different levels in the cord.

5. The ventral roots of the trunk of *Eleutherodactylus* are wholly separate and distinct from the longitudinal motor-tract, the fibres of which they cross at right angles. The arrangement of the ventral roots of the tail resembles that in other Anura.

6. In embryos of the lizard *Sphaerodactylus* the ventral-root fibres arise directly from cells of the basal plate of the cord and run at various angles through the white matter to emerge as spinal motor-roots. In the chick embryo ventral-root fibres run directly at right angles through the white matter.

7. On the basis of observations on the behaviour of nerve fibres in tissue culture, it is suggested that there is a general correlation between the angle of approach of two growing fibres and their chances of permanent anastomosis.

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I am deeply grateful to Dr. Philip Whiting for giving up his time to discuss neuro-embryological problems with me, to Dr. Peter Lewis for allowing me to cite observations yet to be published, and to Dr. R. Sims for much help. My indebtedness to West Indian helpers, acknowledged in a previous paper, remains unrequitable. The expenses of this investigation were partly met by a grant from the Nuffield Foundation to the School of Anatomy, Cambridge. My visit to Jamaica was made possible by a travel grant from the British Council, and by a subsistence allowance from the University College of the West Indies.

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EXPLANATION OF PLATES

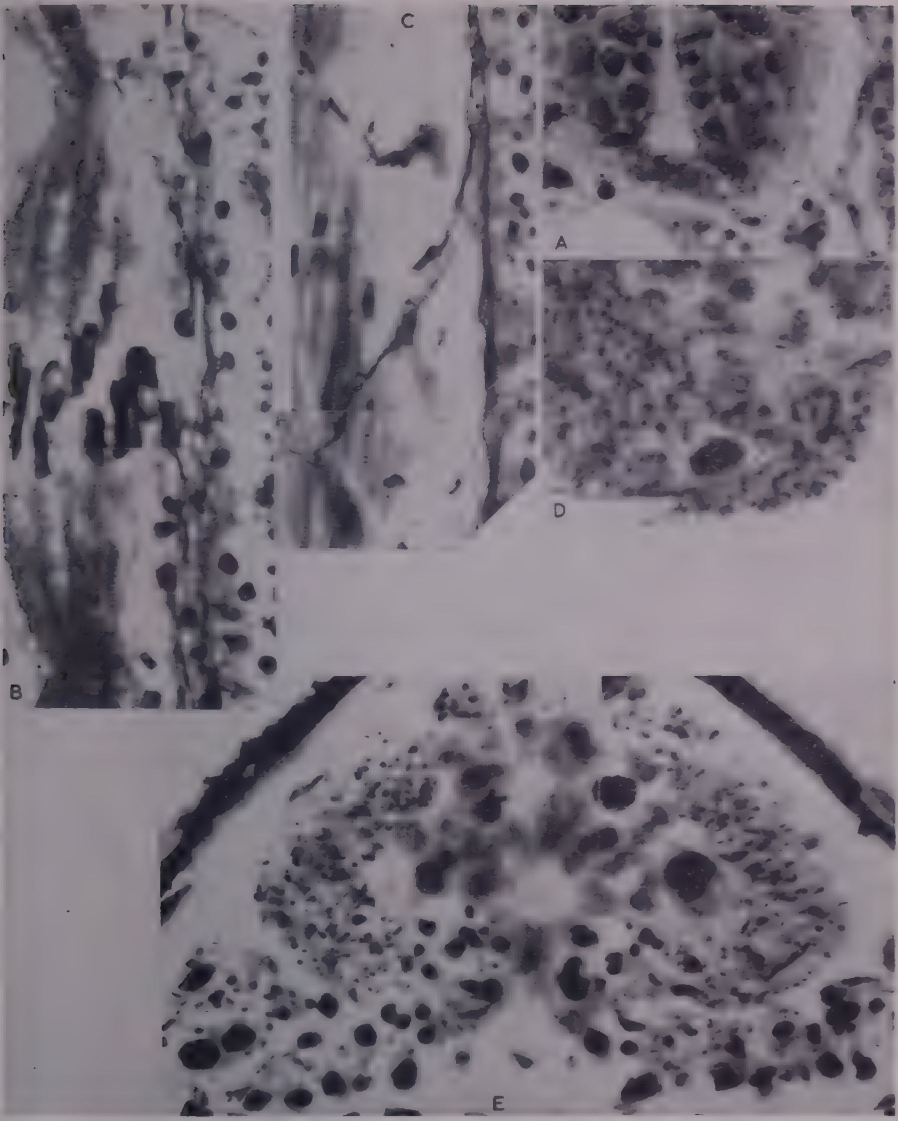
PLATE 1. Spinal motor-roots in *Xenopus laevis*

FIG. A. Transverse section through spinal cord at stage N. and F. 32, showing the hindmost ventral root (right), at a level about two-thirds along the body, where a layer of white matter several fibres thick already covers the ventro-lateral surface of the cord. Holmes. × 530.

FIGS. B and C are from a horizontal section through the cord and myotomes at stage N. and F. 34. Basal plate of cord to right of figure. Bodian. × 530.

FIG. B. In anterior trunk region. One ventral-root fibre runs forward to a myocomma; the next septum receives a backwardly directed fibre from the longitudinal tract.

FIG. C. Posterior trunk region, showing backwardly directed ventral root.



A. HUGHES

Plate 1

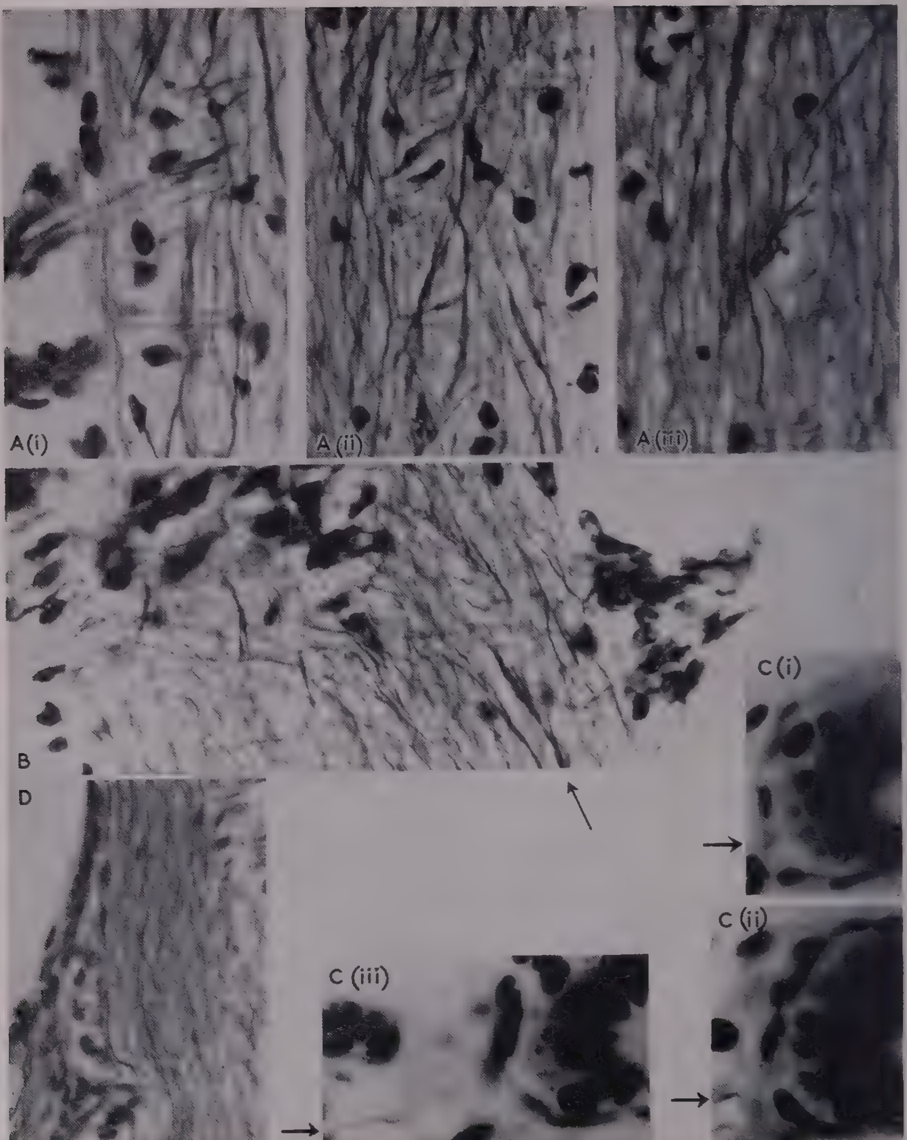


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Plate 2

Figs. D and E. Transverse sections through the cord at stage N. and F. 52.

Fig. D. At level near 4th spinal ganglion. Median ventral sulcus to right of figure. Deep ventral-root fibre sweeps round Mauthner axon (lower centre of figure), and emerges from cord on left × 590.

Fig. E. In tail region. On lower border of figure, ventral roots containing large axons given off from ventro-medial longitudinal tract. Bodian. × 520.

Plate 2

Fig. A. Three successive horizontal sections through trunk cord of larva of *Bufo marinus*. In (i), ventral root (left) is given off. In (ii) and (iii), the constituent fibres can be traced inwards until they merge with the longitudinal tract. Bodian. × 630.

Fig. B. Horizontal section through the trunk cord of an embryo of *Eleutherodactylus ricordii* on the day before hatching, showing the emergence of a ventral root (right). Some of the constituent fibres can be seen entering the white matter (left). The arrow shows the direction of the long axis. Bodian. × 550.

Fig. C (i–iii). Consecutive transverse section through the tail of an embryo of *Eleutherodactylus ricordii* at −6 days, showing the origin of an oblique ventral-root fibre (indicated by arrows). Central canal of cord on right of figure. Bodian. × 870.

Fig. D. Longitudinal section through anterior region of trunk cord of late embryo of *Sphaerodactylus* showing emergence of ventral root (left). Bodian. × 470.

(Manuscript received 19 : viii : 58)

The Adhesive Properties and Expansion of the Chick Blastoderm

by D. A. T. NEW[1]

From the Department of Anatomy, University College London

WITH ONE PLATE

INTRODUCTION

DURING the first 4 days of incubation the chick blastoderm expands to surround the yolk. Its expansion takes place over the inner surface of the vitelline membrane, and the edge of the blastoderm is firmly attached to this membrane.

Little attention has been paid hitherto to the mechanism of this expansion, presumably because it lies outside the embryo proper. But many of the problems involved are of considerable interest, not only as they relate to development within cleidoic eggs, but also in connexion with more general questions affecting expansion of epithelia and the nature of cell surfaces. Blastoderm expansion has many points of similarity with the spreading of epithelia across wounds, and some of the factors involved may prove to be similar to those affecting the radiation of loose sheets of cells in tissue culture. The manner in which the blastoderm edge follows the surface of the vitelline membrane suggests also that it may provide further evidence in support of the views of Holtfreter (1939) and Weiss (1946) on the morphogenetic importance of cell surface structure.

In addition, the chick blastoderm provides excellent experimental material. It is only a few cells thick, expands rapidly, is large enough to be easily handled, and can easily be observed and operated upon *in vitro*. Only the edge of the blastoderm adheres firmly to the vitelline membrane, and if required it can easily be released, reattached or dissected, and the parts rearranged.

The work described here has been aimed at finding answers to the following four questions: (1) Is attachment of the blastoderm edge necessary for expansion? (2) What features of the vitelline membrane affect blastoderm expansion? (3) Why does only the edge of the blastoderm adhere to the vitelline membrane? (4) By what mechanism does blastoderm attachment affect expansion?

It will be convenient to describe the work in four sections corresponding to these questions. Except where stated otherwise, all the *in vitro* work was carried out on blastoderms explanted using the ring technique (New, 1955). This con-

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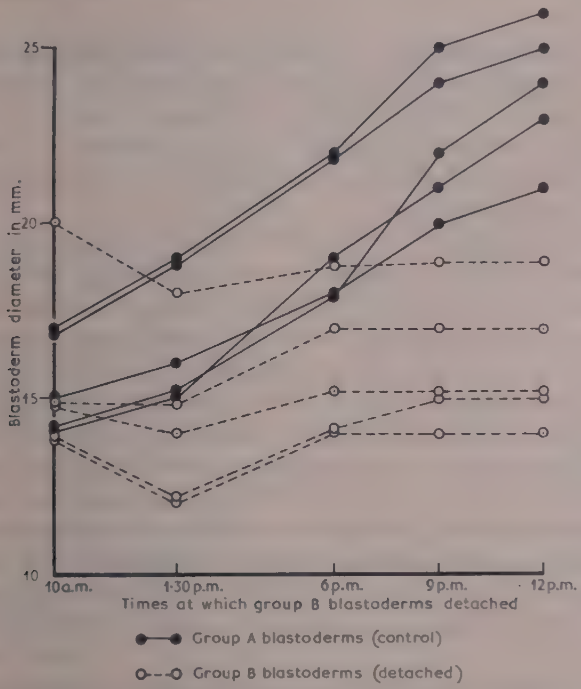
sists essentially of explanting the blastoderm on to a piece of vitelline membrane stretched across a glass ring in a watch-glass containing albumen. The watch-glass is placed inside a Petri dish containing a little water and cotton wool; this acts as a moist chamber.

IS ATTACHMENT OF THE BLASTODERM EDGE NECESSARY FOR EXPANSION?

In this experiment a group of blastoderms explanted by the ring method were freed at the edge from the vitelline membrane every few hours. The changes in diameter of these detached blastoderms were compared with those of a control group.



TEXT-FIG. 1. Expansion of normal and detached blastoderms.

Ten blastoderms were explanted from eggs incubated for about 24 hours. At 36 hours of incubation they were divided into two groups of five, each group containing a similar range of developmental stages (head fold to four somites).

The diameters of all the blastoderms were measured with calipers to the nearest millimetre, and the edge of each blastoderm in one group (Text-fig. 1, Group B) was gently freed from the vitelline membrane with a small scalpel. Care was taken to disturb the rest of the blastoderm as little as possible; and to avoid difficulties arising from surface tension, each preparation was flooded with saline during the operation. The control group (Group A) was removed from the incubator for the same length of time as Group B, and saline was added to and removed from each preparation exactly as in Group B.

The Group B blastoderms were first detached at 10 a.m. and the treatment was then repeated at 1.30 p.m., 6.0 p.m., and 9.0 p.m. On each of these occasions the blastoderms were found to have become reattached, and were freed again. Also the blastoderm diameters were measured at each of these times, and a final measurement was made at 12.0 p.m. These results are shown in Text-fig. 1.

The measurements show that only in the control group has any marked expansion of the blastoderm taken place. The detached blastoderms have shown practically no expansion except during the comparatively long interval between 1.30 p.m. and 6.0 p.m. Following the first treatment they have actually contracted slightly.

The most probable explanation of these results is that the blastoderm can only expand to any great extent if it is attached at the edge to the vitelline membrane. When the edge is freed it requires at least 2 or 3 hours to become fully reattached, and consequently by freeing it every 3 hours, expansion can practically be brought to a standstill. If a much longer period than 3 hours is allowed, however, some expansion will take place.

It might be argued that it is not so much the state of being detached that prevents expansion as injury caused to the blastoderm edge during detachment. This seems extremely unlikely. If there is any injury it must be very small; the blastoderm edge comes away from the vitelline membrane quite easily and there is no obvious tearing or macroscopic damage. Furthermore, this hypothetical small-scale injury must be limited to the extreme edge of the blastoderm since this is the only part touched during the operation; it would only seem to have a chance of being effective in hindering expansion if expansion took place by continuous addition of cells at the extreme edge. But there is no reason to suppose that expansion does occur in this way; in fact, mitotic figures are found far more abundantly at some distance inside the blastoderm than at the extreme edge. Other possible mechanisms of expansion, such as flattening of cells, are even less likely to be affected by small injuries at the extreme edge of the blastoderm.

During explantation of a blastoderm on to a vitelline membrane, too much fluid has occasionally been left on the membrane, so that parts of the blastoderm edge have been floating in the fluid and have therefore been unable to make contact with the membrane. In such blastoderms, even after 12–24 hours, those parts of the blastoderm edge which have failed to contact the membrane remain unexpanded, whilst the parts which have made contact have adhered and show marked expansion.

It seems reasonable to conclude, therefore, that normal expansion of the blastoderm is dependent on an adhesion between the edge of the blastoderm and the vitelline membrane.

WHICH FEATURES OF THE VITELLINE MEMBRANE AFFECT BLASTODERM EXPANSION?

Growth on treated vitelline membranes

Blastoderms explanted on to vitelline membranes expand at the same rate as *in ovo* (New, 1955), but when explanted on to plasma clot surfaces (technique of Waddington, 1932) normal expansion ceases. Since both substrates provide flat, smooth, moist surfaces, the results suggest that the blastoderm is sensitive to chemical differences between the two, and, since both substrates are mainly protein, that the blastoderm edge may be able to distinguish between different types or arrangements of protein molecules.

TABLE 1

Blastoderm expansion on membranes treated in various ways

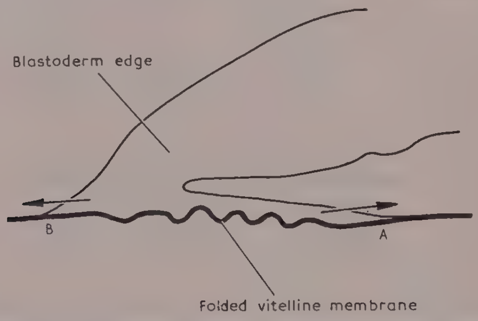
Treatment of vitelline membrane	*Blastoderm diameter at explantation*	*Blastoderm diameter after approx. 16 hours* in vitro	*Blastoderm expansion*	*Adhesion between blastoderm and vitelline membrane*
	(mm.)	(mm.)	(mm.)	
20 min. in 70 per cent. alcohol	10·5	21·0	+10·5	Normal*
	11·0	24·0	+13·0	
20 min. in 4 per cent. formalin	11·0	11·0	nil	Weak
	12·0	11·0	−1·0	
	11·0	17·0	+6·0	Normal
20 min. in boiling saline	10·0	12·0	+2·0	Weak
	9·0	12·0	+3·0	
Vit. memb. dried for 2 hours at 38° C. on a glass coverslip (Text-fig. 3)	11·5	11·5	nil	Weakly attached over whole ectoderm surface
	12·0	<14·0	<+2·0	
	10·0	<13·0	<+3·0	
Dried for 4 hours at 36° C. Placed on a piece of dialysing membrane for support (Text-fig. 3)	9·5	11·5	+2·0	Weakly attached over whole ectoderm surface
	9·5	10·0	+0·5	
Untreated. Normal controls	7·5	18·0	+10·5	Normal*
	13·0	24·0	+11·0	

* The normal condition is a moderately strong adhesion of the blastoderm at the edge only. The rest of the blastoderm is unattached.

To confirm that the blastoderms have such sensitivity, attempts were made to grow them on vitelline membranes treated to denature the proteins. (Note that in all these preparations the blastoderm was placed on that surface of the vitelline membrane to which it would normally be attached *in ovo*, i.e. the surface that would be the *inner* one *in ovo*.) Table 1 summarizes the result of this experiment, and clearly shows that the ability of the vitelline membrane to support blastoderm expansion is easily destroyed. Of the treatments given only alcohol has left the vitelline membrane unimpaired as a substrate. The variation in strength of the adhesion between blastoderm and vitelline membrane is also interesting. 'Strength of adhesion' is a difficult quantity to measure precisely; nevertheless, it is quite possible to detect it when the adhesion is markedly stronger or weaker than normal. As the table shows, abnormality of expansion is correlated with abnormality of adhesive strength.

Growth on inverted vitelline membranes

Explantation of blastoderms on to inverted vitelline membranes (i.e. on to the surface of the vitelline membrane that would be the *outer* one *in ovo*) indicated a marked difference between the two surfaces of the membrane. Thirty-one blastoderms were explanted in this way (after 1 day of incubation *in ovo*) and all behaved similarly; they showed practically no expansion (in fact many contracted slightly; see Tables 3 and 4) and an exceedingly strong adhesion to the vitelline membrane. The adhesion was so firm that it was difficult to release the blastoderms without damaging them.

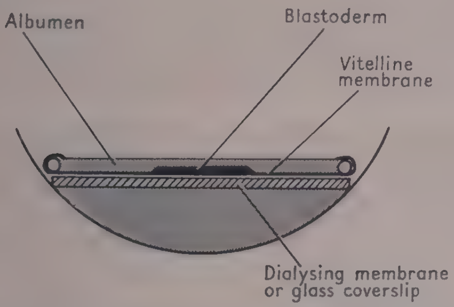


TEXT-FIG. 2. Outline drawing of section through blastoderm edge attached to inverted vitelline membrane (see Plate, fig. C). The arrows show direction in which it is suggested the cells at *A* and *B* may be attempting to move, resulting in a pull on the vitelline membrane (in the *opposite* direction to the arrows) producing folds in the membrane.

At the region of attachment the vitelline membrane could be seen with the naked eye to be thrown into minute ridges. This is shown more clearly in section (Plate, fig. C), where it can also be seen that a thin layer of cells from the blastoderm edge has moved inwards under the blastoderm (at *A*, Text-fig. 2) producing a wide band of attachment between blastoderm and vitelline membrane. The folding of the vitelline membrane corresponds exactly with this area of attachment, and suggests that the cells at *A* and *B* (Text-fig. 2) are exerting a pull on the vitelline membrane. This will be discussed later.

Effects of tension within the vitelline membrane

During the experiments with treated vitelline membranes, blastoderms were explanted on to dried vitelline membrane supported by dialysing tubing (Text-fig. 3). As a control for this a culture was set up in which untreated vitelline membrane was supported by dialysing tubing. Rather surprisingly, the blastoderm on this control vitelline membrane, as well as those on the dried vitelline membranes, failed to expand.



TEXT-FIG. 3. Arrangement of culture when vitelline membrane is supported by glass coverslip or dialysing membrane.

TABLE 2

Effect on blastoderm expansion of supporting the vitelline membrane on glass

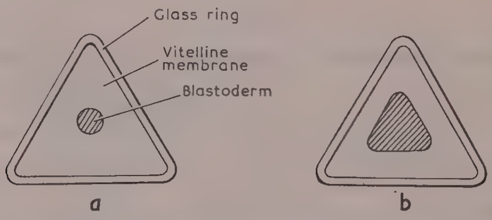
	Blastoderm diameter at explantation	*Blastoderm diameter after approx. 15 hours in vitro*	*Increase of diameter*
	(mm.)	(mm.)	(mm.)
Vitelline membrane over glass	18·5	21·5	3·0
	12·0	16·0	4·0
	10·0	12·0*	2·0
	12·5	23·0†	10·5
	12·0	12·5*	0·5
	17·5	18·0*	0·5
	12·0	13·0	1·0
	15·0	16·0*	1·0
	17·5	19·0	1·5
	15·0	15·0	nil
Normal control. Vitelline membrane over albumen	16·0	21·5	5·5
	15·5	22·5	7·0
	11·0	21·5	10·5
	13·5	18·0	4·5
	18·5	23·0	4·5

* Blastoderm found to be partly detached from vitelline membrane.
† An accumulation of fluid found between vitelline membrane and glass.

This result suggested that it might be necessary for the vitelline membrane to 'hang free' if blastoderm expansion was to occur. To investigate this further ten blastoderms were explanted on to vitelline membranes lying on glass coverslips (Text-fig. 3). The results, together with five controls, are shown in Table 2. (All diameters measured with calipers to the nearest 0·5 mm.)

Many of the blastoderms lying on membrane-over-coverslip failed to show normal expansion, and the fact that four became partly detached from the vitelline membrane suggests that adhesion had also been affected. The blastoderm in this group showing maximum expansion (10·5 mm.) was found to be in a culture where a layer of fluid had been trapped between coverslip and vitelline membrane.

A possible explanation for these results is that the blastoderm is sensitive to lines of mechanical tension within the vitelline membrane, and these lines of tension are upset by contact with a solid surface. To test the sensitivity of blastoderms to lines of tension in the membrane, some blastoderms were explanted on to vitelline membranes stretched across triangular (Text-fig. 4) and square glass rings. These rings were fairly large so that the vitelline membrane had to be stretched tightly to make it extend to the angles of the ring; thus there were lines of tension radiating out from the angles.



TEXT-FIG. 4. A circular blastoderm (*a*) placed on vitelline membrane stretched across a triangular ring expands in the form of a triangle (*b*).

Two circular 1-day incubated blastoderms were explanted into square ring cultures. After 2 hours of *in vitro* culture, expansion of the blastoderms towards the angles of the square was found to be greater than that towards the sides. (Diagonal measurements of blastoderm A, 21·5 mm. × 20 mm.; of blastoderm B, 17 mm. × 16 mm. Side to side measurements of blastoderm A, 18·5 mm. × 17 mm.; of blastoderm B, 16 mm. × 15·5 mm.)

Three circular 1-day incubated blastoderms were explanted into triangular ring cultures. After 20 hours of *in vitro* culture the blastoderms had become markedly triangular in shape (Text-fig. 4, and Plate, fig. D) with the angles of the blastoderm extending towards the angles of the ring. In a repeat of this experiment, triangular blastoderms were removed from the vitelline membranes and the membranes examined under the polarizing microscope, but no birefringence could be detected.

Evidently the blastoderm edge is sensitive to lines of tension within the vitelline membrane. But is the vitelline membrane normally under tension? There seems little doubt that it is. If the vitelline membrane in a watch-glass culture is slackened so that wrinkles appear in it, and the preparation is then returned to the incubator, the wrinkles gradually disappear and the membrane becomes smooth again. Evidently the membrane has a slow spontaneous contractility which, provided it is attached to a rigid frame, will bring it into a condition of tension even though none may have been imposed initially.

If tension is normally present in the vitelline membrane, and the blastoderm is sensitive to membrane tension, it seems likely that anything that interferes with the pattern of membrane tension will also interfere with blastoderm expansion. Therefore, the reason why vitelline membranes in contact with solid surfaces fail to support normal blastoderm expansion is likely to be that such contact prevents the formation of a normal pattern of tensions in the membrane. However, there are at least two other possible explanations. (1) The membrane-over-coverslip cultures might leave the blastoderms short of nutrient materials. (In these and in the control cultures a thin layer of albumen was placed over the blastoderm, but the controls could probably also use nutrient substances from underneath the vitelline membrane.) (2) Contact with a glass or dialysis tubing surface might affect the chemical composition of the vitelline membrane. At present these alternative explanations cannot be excluded.

The experiments described in this section indicate that the blastoderm is sensitive both to the chemical structure of the vitelline membrane surface and to lines of tension within the membrane; they also suggest that both these features of the vitelline membrane can easily be impaired. The implications of this will be left to the discussion at the end of the paper.

WHY DOES ONLY THE EDGE OF THE BLASTODERM ADHERE FIRMLY TO THE VITELLINE MEMBRANE?

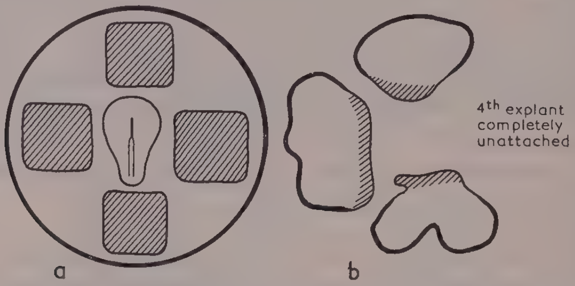
Only the extreme edge of the blastoderm adheres strongly to the vitelline membrane. The remainder is either unattached or possibly very lightly attached —the difference is difficult to detect—but as its exact condition is irrelevant to the present work it will simply be referred to as ‘unattached’.

What causes the edge to be attached whilst the rest is unattached? As a first step towards analysing this question the following two hypotheses have been considered. (1) The cells of the blastoderm edge are intrinsically different as regards their surface structure from those of the rest of the blastoderm. (2) The cells of the blastoderm edge are intrinsically the same as those of the rest of the blastoderm as regards their surface structure, but behave differently as a result of their position, i.e. their position at the edge brings them under some influence, either environmental or blastodermic in origin, which causes them alone to adhere to the vitelline membrane.

If the second hypothesis is correct then centrally placed cells should, if released from their neighbours (so that they lie on an artificially created edge), prove capable of adhering to the vitelline membrane; and, conversely, two confronted blastoderm edges if given the opportunity to fuse together should lose their attachment to the vitelline membrane along the line of fusion. Both these experiments are described below.

Behaviour of detached parts of the blastoderm

Six blastoderms from eggs incubated for about 24 hours were explanted using the ring technique. Explants were cut from the area opaca of each blastoderm and an outline drawing made in each case to record the position from which the explant was obtained. The procedure is illustrated in Text-fig. 5*a*. Only the explants were retained, the remainder of the blastoderm being detached from the vitelline membrane and discarded.



TEXT-FIG. 5. Four explants were made from a blastoderm (*a*) (in redrawing, the distance of explant from blastoderm edge has been exaggerated—Editor). On further incubation three of these explants attached themselves to the vitelline membrane and expanded (*b*). Edges attached to the vitelline membrane are shown by thick lines.

It will be noted that although the explants were cut so that in each case one edge was situated in the extreme peripheral region of the blastoderm, the whole perimeter of the explant has been formed by cutting. Any difference in behaviour between its edges cannot, therefore, be attributed to differences of injury.

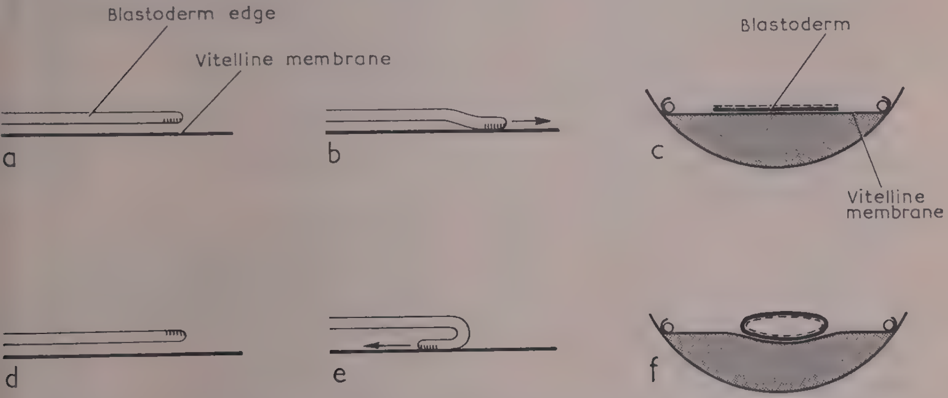
Care was taken to see that the explants were completely free of the vitelline membrane, and the preparations were returned to the incubator for a further 16 hours. The results in practically all cases were very similar, and Text-fig. 5 shows a typical example. The outer edge (i.e. the peripheral blastoderm) of the explants became firmly attached to the vitelline membrane, but the inner edges did not. The explants do not, of course, retain their original size and shape, since expansion continues as soon as attachment occurs.

This experiment therefore suggests that the property of adhesion to the vitelline membrane shown by the edge of the blastoderm depends on the intrinsic nature of the blastoderm material of this region, and not on its position.

Behaviour of confronted blastoderms

Eight blastoderms were freed from the vitelline membrane and arranged into four cultures, each containing two blastoderms on the same membrane. The distance between each pair of blastoderms was 2–3 mm. The cultures were returned to the incubator and examined at intervals during the next 24 hours.

All four cultures gave similar results. The two blastoderms of each pair reattached to the vitelline membrane and expanded until the advancing edges met. Fusion then occurred. Expansion along the line of fusion ceased, but continued around the remainder of the peripheries. The strength of adherence between the two blastoderm edges was found to be very great. A strong enough pull on one blastoderm would tear the other away from the vitelline membrane without breaking the two blastoderms apart. But although the confronted edges had fused, they still retained a firm attachment to the vitelline membrane. Again, this suggests an intrinsic difference in surface structure between the edge cells and the remaining cells of the blastoderm.



TEXT-FIG. 6. *a*, *b*, *c*, behaviour of blastoderm explanted normally on to vitelline membrane. Adhesive surface attaches to the vitelline membrane (*b*). Normal expansion follows as shown by diagram of whole preparation (*c*). *d*, *e*, *f*, behaviour of blastoderm inverted on vitelline membrane. Blastoderm edge curls under to bring adhesive surface against vitelline membrane (*e*). Expansion in this case results in formation of a hollow vesicle (*f*; see also Plate, fig. E). (In *c* and *f* thick line denotes ectoderm, broken line endoderm. In *a*, *b*, *d*, and *e* shading indicates adhesive surface.)

It is clear from these experiments that the first hypothesis is correct and that the second one is not. The edge of the blastoderm has a surface structure peculiar to this region. It will be convenient to refer to this special surface as the 'adhesive surface'.

Exactly how far this adhesive surface extends inwards from the edge has not been determined, though the area of a normal blastoderm which is actually attached to the vitelline membrane is certainly smaller than the yolk-free peripheral region usually termed the 'margin of overgrowth'. Furthermore, experiments with inverted blastoderms (New, 1956) have suggested that the adhesive surface only occurs on one face of the blastoderm edge (Text-fig. 6*a*). If a blastoderm is explanted with its endoderm face against the vitelline membrane, instead of attaching and expanding normally, the edges curl under and grow towards each other (Text-fig. 6 *d*, *e*, *f*). Eventually the edges fuse and form a hollow vesicle (Plate, fig. E). The simplest explanation for this is that the face of the blastoderm edge that would normally lie against the yolk lacks any adhesive surface, so that in the inverted blastoderms attachment to the vitelline membrane can only occur where the edge has curled under. It is not necessary to suppose that the whole edge spontaneously curls under; provided it happens at one point, this will attach and gradually pull the rest under. Such starting-points are very likely to be formed fortuitously when the blastoderm is being explanted.

BY WHAT MECHANISM DOES ATTACHMENT AFFECT EXPANSION?

It is evident from the results given in the first section that expansion of the blastoderm can only occur if its edge is attached to the vitelline membrane. A probable explanation of this is that the outward movement of the attached edge creates a tension within the blastoderm, and this tension then causes expansion.

The existence of such a tension is illustrated by the following:

(i) In Text-fig. 1 the measurements given of Group B blastoderms at 1.30 p.m. suggest that the blastoderms have contracted slightly in diameter after being freed initially from the vitelline membrane.

(ii) The appearance of the expanded explants in Text-fig. 5 suggests that expansion has only occurred in regions lying between attached edges. These are obviously the only regions that could be in a state of mechanical tension. In the neighbourhood of unattached edges little expansion seems to have occurred; this was judged by the density of yolk granules in these regions, which left the tissue (shaded in the drawing) almost as opaque as the area opaca of the blastoderms from which the explants were taken.

(iii) Detached blastoderms frequently curl up at the edges. This suggests mechanical tensions which are stronger peripherally.

(iv) After treatment with mitotic inhibitors (Bellairs, 1954) expansion of the blastoderm continues at about the normal rate, but numerous spaces appear in it. Apparently in the absence of sufficient cells to cover the whole area, a tension is set up by the outgoing edge strong enough to tear the blastoderm.

It is, of course, just possible that attachment of the blastoderm edge is necessary for expansion for reasons other than the production of tension. But it is difficult to think of a reasonable alternative. Furthermore, if tension is not necessary, one would expect instances where rate of expansion had overtaken the rate of movement of the attached edge (e.g. where the latter is stationary, as it is on an inverted vitelline membrane); but this has never been observed.

Assuming, therefore, that it is the mechanical tension in the blastoderm that causes expansion, how does it act? The question has been considered at two levels: the possible effect of tension on growth (i.e. protoplasm synthesis), and the possible effect of tension on cell division and arrangement.

Effect of tension on growth

Comparisons were made between blastoderms explanted normally on vitelline membranes and blastoderms explanted on to inverted vitelline membranes. In the latter there is no outward movement of the edge and therefore, presumably, no tension (except possibly in a very small region at the extreme edge—see discussion). As a measure of protoplasm synthesis nitrogen (N) determinations were made by a micro-Kjeldahl method.

TABLE 3

Comparisons of nitrogen content of blastoderms incubated 24 hours in ovo *with blastoderms incubated 24 hours* in ovo *followed by 17 hours* in vitro, *some explanted normally, others on inverted membranes*

	Blastoderm diameter at explantation (after 24 hours incubation in ovo)	*Blastoderm diameter after 17 hours* in vitro	*Nitrogen (as c.c. of N/100 ammonia)*
	(mm.)	(mm.)	
Explanted normally	7·0	19·0	0·61
	10·0	21·0	0·85
	9·0	19·5	0·88
	10·5	20·5	1·27
	10·5	20·0	1·24
	10·5	22·0	1·05
	11·0	26·0	1·24
	11·0	26·0	1·28
	10·5	24·5	1·52
	9·0	18·0	1·23
	10·0	25·0	1·00
Mean and standard error			1·106±0·076
Explanted on inverted vitelline membrane	10·0	9·5	0·61
	10·5	9·5	0·51
	8·0	6·0	0·38
	9·0	8·5	0·52
	5·5	4·5	0·17
	6·0	6·5	0·35
	7·0	7·5	0·41
	13·5	?*	0·75
	9·0	9·0	0·41
Mean and standard error			0·457±0·052
No incubation *in vitro*	12·0		0·71
	8·5		0·52
	9·5		0·24
	11·5		0·46
	8·5		0·15
	9·5		0·28
	11·0		0·61
	9·0		0·39
Mean and standard error			0·420±0·063

* Blastoderm largely detached from vitelline membrane and curled up.

Table 3 summarizes the results of such an experiment. Twenty-eight blastoderms from eggs incubated for 24 hours were divided into three groups. The first group of eleven blastoderms was incubated in a normal *in vitro* culture for 17 hours and then estimated for N. The second group of nine blastoderms was also incubated *in vitro* for 17 hours, but on inverted vitelline membranes. The third group of eight blastoderms was estimated for N immediately after explantation from the egg.

TABLE 4

Nitrogen content of blastoderms cultured as in Table 3 but after blastoderm and vitelline membrane had been cleaned of yolk

	Blastoderm diameter at explantation (after 24 hours incubation in ovo)	*Blastoderm diameter after 17 hours* in vitro	*Nitrogen (as c.c. of N/100 ammonia)*
	(mm.)	(mm.)	(mm.)
Explanted normally	5·5	14·0	0·41
	7·5	20·0	0·67
	8·0	19·0	0·54
	8·5	13·5	0·62
	9·5	20·0	0·71
	13·0	19·5	1·27
	10·5	19·0	0·61
	9·0	18·5	0·63
Mean and standard error			0·682±0·079
Explanted on inverted vitelline membrane	9·5	7·0	0·69
	9·5	8·0	0·71
	6·0	4·5	0·23
	12·5	10·0	1·07
	8·5	6·5	0·49
	12·0	10·5	0·98
	8·5	7·0	0·46
	5·0	5·0	0·21
Mean and standard error			0·605±0·105
No incubation *in vitro*	8·5		0·50
	10·5		0·73
	7·0		0·48
	11·0		1·29
	10·0		0·82
	9·5		0·64
	12·5		0·90
	7·5		0·46
	7·0		0·37
Mean and standard error			0·688±0·090

Comparison of the normally explanted group with the unincubated *in vitro* group shows a highly significant increase in nitrogen content ($P = < 0{\cdot}001$). But the difference in nitrogen content between the inverted vitelline membrane group and the unincubated *in vitro* group is insignificant (*P* is between 0·6 and 0·7). This suggests that synthesis of protoplasm in the blastoderm ceases almost entirely, or entirely, unless the blastoderm edge is able to move outwards.

This conclusion would only be valid, however, if the blastoderm were able to utilize albumen protein. If it were not, then its source of nitrogen would be restricted to the small amount of yolk adhering to the upper surface of the vitelline membrane. Only the normally explanted blastoderms with their outward-pulling edge would have the possibility of picking up these fragments of yolk, and therefore the difference in amount of synthesis shown by the two groups of explanted blastoderms could be accounted for simply by localization of food-supply.

To avoid this ambiguity the experiment was repeated using vitelline membranes and blastoderms carefully washed clean of all adherent yolk. The results of this are shown in Table 4. Neither the inverted vitelline membrane group nor the normally explanted group shows a significant increase in nitrogen content. Comparison of the inverted vitelline membrane group with the unincubated *in vitro* group gives P between 0·8 and 0·9; the normally explanted compared with the unincubated *in vitro* group gives $P = > 0{\cdot}9$. (The eggs used in the experiment recorded in Table 3 were obtained in November and early December. Those used for the experiment recorded in Table 4 were obtained in late January. This probably accounts for the higher N content of the non-incubated group in Table 4 even after cleaning off adherent yolk. The 'uncleaned' blastoderms of Table 3 would in any case be left fairly clean.)

Evidently albumen nitrogen is not utilized by blastoderms of this stage, so that the differences of nitrogen uptake shown in Table 3 must represent differences of yolk utilization. Where no yolk is available (see Table 4), neither group of cultured blastoderms shows protoplasm synthesis.

But Table 4 shows most strikingly that even without any synthesis of protoplasm a great deal of blastoderm expansion can occur provided the blastoderm edge is able to move outwards; the normally explanted blastoderms have doubled or trebled their diameter. Whilst, therefore, the possibility of a direct effect of tension on protoplasm synthesis cannot be completely ruled out, it seems likely that the main primary effect of tension is to stretch the blastoderm over a greater area, and any increase in rate of protoplasm synthesis is a secondary effect resulting from increase in absorptive surface.

Effect of tension on cell division and arrangement

If the outward movement of the blastoderm edge is prevented, does cell division within the blastoderm cease, or does it continue with a consequent crowding of cells?

A comparison was made between blastoderms fixed and sectioned after (*a*) 1-day incubation in ovo, (*b*) 1 day *in ovo* followed by 16 hours *in vitro* (normal ring preparation), and (*c*) 1 day *in ovo* followed by 16 hours *in vitro* on inverted vitelline membranes (no blastoderm expansion). A typical section from a blastoderm of each group is shown in the Plate, figs. A, B, C.

The sections show that the cells are much more crowded in the group (*c*) blastoderms than in those of the other two groups. This is particularly clearly seen in the ectoderm. The ectoderm of the expanded blastoderms and of the unincubated *in vitro* blastoderms is one, rarely two, cells thick. The ectoderm towards the margin of the group (*c*) blastoderms is in many places three or even four cells thick, and also shows some slight tendency to fold. (More centrally in the area opaca of group (*c*) blastoderms the thickening is less marked, suggesting that most of the cell division takes place towards the margin.)

Evidently cell division continues in the absence of movement of the blastoderm edge, but the cells are unable to arrange themselves into flat sheets unless the moving edge creates a tension. It appears unlikely that the edge has any direct effect on cell division though it will, of course, affect it indirectly by stretching the blastoderm over a greater area and hence by increasing the absorption of food materials from the yolk.

DISCUSSION

The work described here particularly emphasizes the importance of the vitelline membrane for expansion (or epiboly) of the chick blastoderm, and also the special 'affinity' which exists between the inner surface of the vitelline membrane and the blastoderm edge.

The blastoderm edge is normally attached to the inner surface of the vitelline membrane and moves over the vitelline membrane surface as the blastoderm expands. Treatment of the vitelline membrane by boiling or with formalin makes it incapable of supporting normal blastoderm expansion. Blastoderms placed on the outer surface of (untreated) vitelline membranes fail to expand, and also fail on glass or plasma clot surfaces. This high specificity of attachment shown by the blastoderm edge suggests strongly the type of 'tissue affinity' discussed by Holtfreter (1939) and Weiss (1946). In the egg it presumably serves to ensure that the blastoderm in its expansion will follow the path laid down by the vitelline membrane, and hence will come to surround the yolk. It is interesting to note that Trinkaus (1951) has produced some evidence of a similar mechanism in *Fundulus*, in which the blastoderm shows an attachment affinity for the periblast and appears to follow the 'path' laid down by the periblast.

Probably the affinity shown by the blastoderm edge for the vitelline membrane depends on a molecular surface structure complementary to that of the vitelline membrane. This particular surface structure seems to be entirely restricted to the edge cells and it is only present on the outer surface of these (see Text-fig. 6). Furthermore, it is evidently quite distinct from the surface structure which binds the cells of the blastoderm to each other—at least as regards the ectoderm. It would be of great interest to know at what stage in development the edge cells acquire this peculiarity, and why they develop differently from the rest of the blastoderm.

Particularly curious is the sensitivity shown by the blastoderm edge to lines of tension within the vitelline membrane. This sensitivity recalls that shown by extending nerve fibres (Weiss, 1955). Two facts have been established: the blastoderm expands more rapidly along lines of greater tension (as shown by growth on vitelline membranes stretched across triangular rings); and normal expansion is seriously upset if the vitelline membrane is lying on a coverslip.

Further experiments will be required before any definite conclusions can be drawn about the mechanism involved here. But it is possible that the problem is related to the contractility shown by the vitelline membrane. If the vitelline membrane in a watch-glass culture is slackened so that wrinkles appear in it, and the preparation is then returned to the incubator, the wrinkles rapidly disappear and the membrane becomes smooth again; evidently the membrane has contracted and is again under tension. It might be this process which is interfered with when the vitelline membrane is resting on a glass coverslip instead of hanging free in a fluid medium; conceivably contact with a rigid surface prevents the formation of the normal tension within the vitelline membrane and the adoption of the molecular pattern necessary for blastoderm expansion. In the egg the vitelline membrane is not in contact with any rigid surface and it is probably kept in a state of tension by its contractility acting against the osmotic pressure of the yolk (the osmotic pressure of the yolk is higher than that of the albumen—see Needham, 1950, pp. 11–14).

Whether the tendency of the blastoderm to expand more rapidly along lines of greater tension has any significance *in ovo* is doubtful. Probably the tensions acting at any point in the vitelline membrane in the egg are the same in all tangential directions. But it is conceivable that there *might* be some polarity, stronger lines of tension running meridianally; the animal–vegetal polarity of the yolk might provide the basis for resisting such a pattern of tensions in the vitelline membrane. If there were stronger lines of tension running from animal to vegetal pole in the vitelline membrane, they might be expected to play an important part as guide lines for the expanding blastoderm. But there is at present no evidence for or against their existence.

When blastoderms are explanted on to inverted vitelline membranes (i.e. on to their outer surfaces) they fail to expand though the edge strongly adheres to the membrane. In section this edge appears quite different from a normal blastoderm edge (Plate, figs. B, C). The area of attachment is large and over this area the vitelline membrane is folded. Moreover, a layer of cells appears to have turned back under the blastoderm (*A* in Text-fig. 2). This strongly suggests that some edge cells are trying to move outwards (at *B*) whilst others are trying to move inwards (at *A*); both groups of cells are pulling on the vitelline membrane, with the result that the membrane between *A* and *B* is thrown into folds. If this interpretation is correct it suggests the possibility that the two surfaces of the vitelline membrane differ in their capacity to indicate direction to the blastoderm edge: the edge cells can adhere to either surface of the vitelline membrane, but only on the inner surface are the right pathways provided to keep the cells moving in one direction only.

The crowding of cells in the marginal regions of unexpanded blastoderms

(Plate, fig. C) suggests that cell division occurs with greater frequency towards the margin of the blastoderm than in more central regions (see pp. 160–1). This supports the view of Schlesinger (1952), based on cauterization experiments, that the blastoderm expands by addition to its margin and not by an outward force exerted by the central region. However, the addition is not, as he supposes, by the progressive formation of new cells round free nuclei lying outside the blastoderm edge. The blastoderm expands perfectly well *in vitro*, where there cannot be any free nuclei. (Schlesinger's explanation of his results is based on the histological work of Olsen (1942); but Olsen's account referred to very young cleavage stage blastoderms, whereas the blastoderms used in Schlesinger's experiments were similar in age to the ones described here, i.e. 1 or 2 days' incubated.) Rather it seems that the region of maximum cell proliferation is just inside the edge (Feulgen stained blastoderms show few, if any, mitotic figures at the extreme edge), and the edge cells themselves are concerned with guiding the expanding blastoderm along the vitelline membrane surface and keeping it in a state of tension. Evidence has been given that such a tension exists, and that this tension is necessary for blastoderm expansion. A similar tension has been shown in the teleost (*Fundulus*) blastoderm, which contracts if its margin is released (Trinkaus, 1951).

The fine processes frequently seen on the edge cells suggest amoeboid movement. These cells continue to move over the vitelline membrane even when cell division in the blastoderm has been prevented (Bellairs, 1954) or when the protein supply has been removed (Table 4). On the other hand, if movement of the edge is prevented from occurring, thickening of parts of the blastoderm is seen. Evidently movement of the edge cells and cell proliferation or protoplasmic synthesis are largely independent of each other. The movement of the blastoderm edge is governed primarily by the nature of the substrate surface, and not by events occurring in the rest of the blastoderm; the edge movement affects nutrition and cell proliferation only in the sense that stretching the blastoderm increases the absorptive area.

What happens when the blastoderm edge reaches the vegetal pole of the vitelline membrane? It can then go no farther and hence must cease to maintain a tension within the blastoderm. Yet blastoderm (i.e. yolk sac + chorion) expansion continues; the edge reaches the vegetal pole on the 4th day of incubation; expansion continues rapidly until the 6th day (Grodzinski, 1934). Probably the necessary tension during this period arises from the rapid formation of sub-blastodermic fluid (New, 1956). This fluid, removed from the albumen and secreted into the yolk sac cavity, steadily increases the volume surrounded by the blastoderm and hence is very likely to maintain the blastoderm in a state of tension.

SUMMARY

1. Chick blastoderms during the first 2 days of incubation are unable to expand if the blastoderm edge is detached from the vitelline membrane. The edge can, however, reattach, and then expansion continues.

2. The blastoderm edge is very sensitive to the nature of the substrate surface. It did not form normal adhesions with, or expand normally over, vitelline membranes that had been boiled or (with one exception) treated with formalin. Neither will it expand over the outer surface of the vitelline membrane (i.e. the surface which *in ovo* faces the albumen). It will, however, expand over alcohol-treated vitelline membranes.

3. The blastoderm is sensitive to lines of tension in the vitelline membrane, extending fastest along lines of greatest tension.

4. The marginal cells of the blastoderm adhere to the vitelline membrane as a result of their intrinsic properties, and not because of their peripheral position.

5. Expansion of the blastoderm is dependent on a mechanical tension produced by the outward-moving margin.

6. Expansion can occur without protein synthesis, but if yolk protein is available it will be utilized. Albumen protein is not used at this stage.

7. Cell proliferation is particularly active in the marginal region of the blastoderm (though not at the extreme edge). The ectoderm in the marginal region becomes several cells thick if blastoderm expansion is prevented.

8. Blastoderms inverted on the vitelline membrane develop into hollow vesicles.

9. Blastoderms grown on inverted vitelline membranes adhere abnormally strongly to the membranes and fold the membranes at the region of attachment.

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATE

A. Section through the edge of a blastoderm incubated for 24 hours *in ovo.* (The blastoderm became detached from the vitelline membrane during fixation and the membrane has been omitted from the photograph.) × 120.

B. Section through the edge of a blastoderm after expansion *in vitro.* Explanted at 24 hours' incubation and grown for 16 hours *in vitro.* (The blastoderm has become detached from the vitelline membrane during fixation.) Blastoderm diameter 21 mm.; with 5-somite embryo. × 120.

C. Section through the edge of a blastoderm after cultivation on an inverted vitelline membrane. Explanted at 30 hours' incubation and grown for 16 hours *in vitro.* The blastoderm has not expanded *in vitro* and the cells have become crowded—particularly towards the edge. Blastoderm diameter 9 mm.; with 10-somite embryo. × 80.

D. Triangular blastoderm developed from a circular blastoderm explanted on to vitelline membrane stretched across a triangular glass ring.

E. Section through a blastoderm grown into a hollow vesicle as a result of explantation endoderm downwards on to the vitelline membrane.

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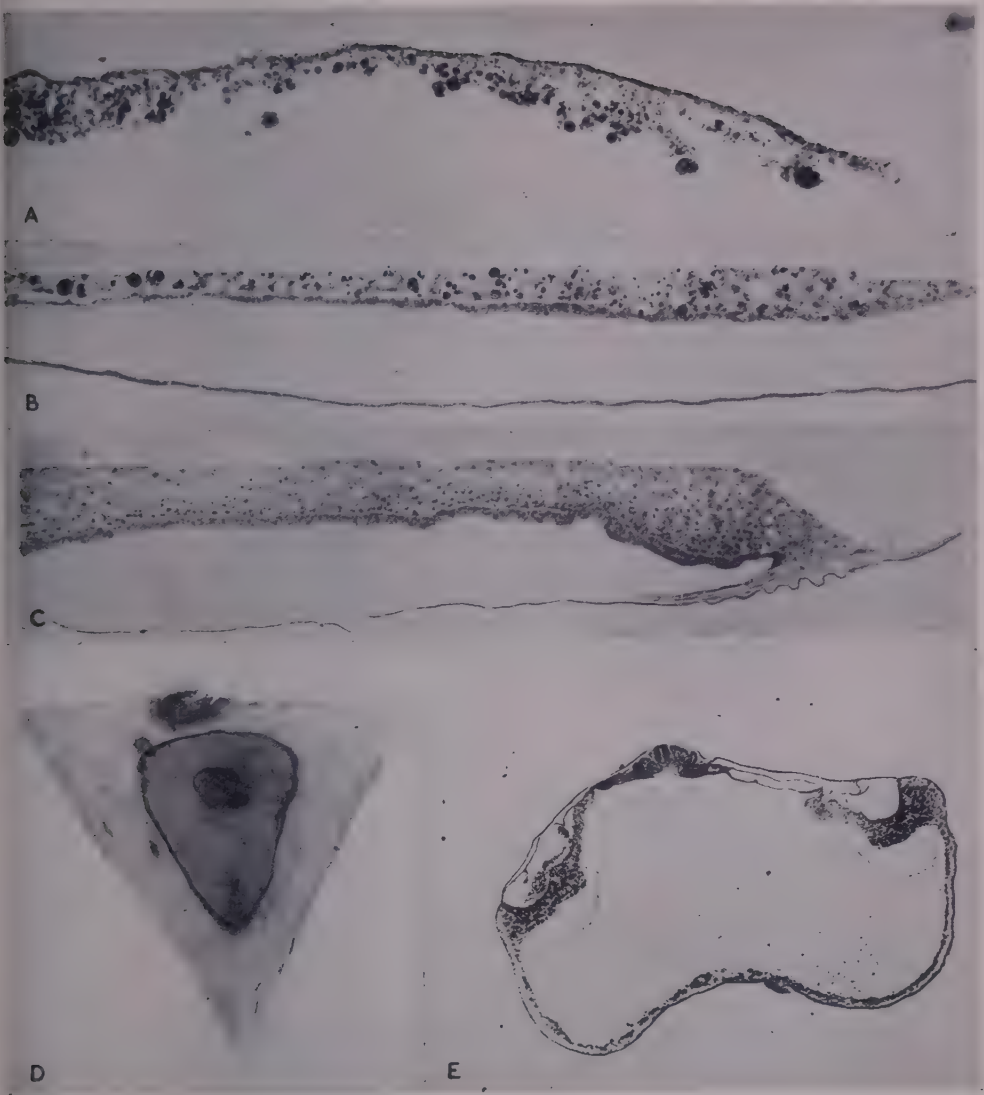

D. A. T. NEW

The Responses of Some Developing Feathers to X-rays

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WITH FOUR PLATES

INTRODUCTION

THE work here to be described was undertaken in order to see, first, whether the treatment of feathers by X-rays would have an effect similar to that described for hair (Chase, 1949, 1951) and, if such an effect were found, then, second, to see whether the results could be more reasonably attributed to a direct effect on the colour-producing cells themselves rather than to an effect upon the epithelium the cells of which would normally receive the pigment. The colours in the normal feathers used are yellow and black, and these are, in the definitive feather, due to the presence of finely divided yellow melanin and black melanin in easily recognizable pillules. The melanin in each case is elaborated in pigment-producing cells such as those shown in Plate 1, figs. A and C. In some colour-producing cells at the base of the normal neck-feather the pigment, which cannot be donated because the cells lack processes, is to be seen aggregated into relatively large masses giving the cell a mulberry-like appearance (Plate 1, fig. D), reminiscent of the condition seen in some melanomata (Plate 1, fig. B) where, again, the pigment apparently cannot be passed on.

One of the objects of the investigation was to see whether these feathers, upon irradiation, showed recognizable changes in the distribution of yellow and black and whether the pigment-producing cells showed any abnormality in the physical condition of their contained pigment. It was also regarded as important to see whether any such effect would persist in a series of feathers from the same follicle.

MATERIAL AND METHODS

Four Brown Leghorn capons, all some years old, were used. They were originally obtained from the Poultry Research Centre at Edinburgh through the courtesy of Dr. A. W. Greenwood. On each bird a patch of about 3 cm. × 2 cm. was plucked on the back of the neck. The patch extended from near the base of the skull downwards. Marks were tattooed on the skin delimiting the area under

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consideration. The plucked feathers appeared normal in every way (Plate 2, fig. A). It was subsequently not always possible to identify the germ from which each feather had grown, partly because the skin shrinks and becomes distorted, and partly because follicles are likely to degenerate and to be replaced by new ones, both changes probably being consequences of irradiation. It was in fact only possible to identify with certainty a few follicles by reference to tattoo marks and by constant examination.

Feathers grew in again after the initial plucking, and when they were a little over half an inch long (after about 2 weeks' growth) each bird was irradiated. The doses given were 100 r., 500 r., 1,000 r., and 2,000 r., all ± 10 per cent. The radiation was directed upon the plucked patch and was of approximately 1·25 mm. aluminium half-value layer; the focus-skin distance was 10 cm. for the three lower doses and 9·2 cm. for the highest dose. In each case pin-feathers from the irradiated patch were then plucked, two each day from the day after irradiation onwards until they were all used up, and then more at longer intervals as new ones grew in again. Some were flattened and mounted permanently, and some were fixed whole and sectioned. All feathers which later grew from the patch in each bird were plucked and filed at intervals over a period of more than two years.

RESULTS

The responses of the tissues apart from changes in pigmentation

All the doses given proved locally traumatic in some degree. On macroscopic inspection local erythema was obvious after a day or two in all the birds. This cleared up quickly in the three birds receiving the lower doses, but in the bird receiving 2,000 r. it became very intense and the whole irradiated patch then became oedematous and greenish. This persisted for about 10 days.

In spite of the erythema and oedema, feather growth, though disturbed, continued in nearly all the follicles in which there had been pin-feathers at the time of irradiation. All the feathers produced in the four birds over a period of over two years were normal in structure, though a little reduced in size in the bird receiving 2,000 r. (Plate 2, fig. B).

The disturbance of the feather growth processes, and their return to normal, were studied in detail in the pin-feathers which were plucked at intervals after irradiation and either flattened or sectioned.

Recognizable disturbances were restricted to the bird receiving 2,000 r. In this bird there were immediate marked abnormalities of growth which lasted for about 10 days, at the end of which period normal growth was re-established in those follicles which had not been permanently destroyed. The ectodermal structures were most obviously affected, but the layers of keratinized cells, both on the cylinder and on the inner aspect of the follicle wall were, as was to be expected, not visibly damaged.

At the very base of the cylinder the living ectoderm began to break down within 24 hours (Plate 3, fig. A). In 2–3 days exudate and broken-down cells filled the space between the follicle wall and the cylinder. The keratinization of the very lowest part of the cylinder had been broken enough to allow the passage of this material through it (Plate 3, fig. B). At about this stage the Malpighian layer above the level of the collar was vacuolated and degenerating, and the tissue which had been beginning to differentiate into barbs was beginning to show signs of disorganization (Plate 3, fig. F).

By the third day the Malpighian layer at the base of the cylinder was recovering and growing up and round the pulp, healing it in and cutting off on its outer side the damaged original Malpighian layer cells and, of course, everything outside them, including the sheath. In this fashion a new cylinder of ectoderm was formed at the base, surrounding the old pulp on its sides but not yet covering it over above. Broken-down ectoderm, barb-rudiments, exudate, and cells presumed to be leucocytes can be seen filling the space between this new cylinder and the inner wall of the follicle (Plate 3, figs. E, F).

By the fourth day this new ectodermal cylinder had formed a complete cap over the mesodermal pulp, keratinized on its upper surface also (Plate 3, fig. C). Since this new cylinder is to give rise to the new feather, the new feather is morphologically within the old one.

The pulp had, without much doubt, remained alive, and it contained engorged blood-vessels. It appeared to be under pressure: the newly growing ectodermal cells embracing it were stretched and elongated parallel to the basement membrane, which was now fully restored. Fibres in the pulp which were normally straight were now wavy, as if too long for the space available to them.

During the next week or so the new cylinder continued to lengthen, and in doing so pushed out all the old pin-feather material. Plate 3, fig. D, shows this 11 days after irradiation. The level of the base of the old pin-feather (clearly seen in the photograph) is marked by the presence of the remains of the old sheath and of the old follicle-lining. At about this time circumferential growth of the cylinder occurs and the barbs and barbules begin to form in it.

The responses of the tissues leading to changes in pigmentation

The macroscopic appearance of the feathers plucked after irradiation

General response. The three birds receiving less than 2,000 r. showed no recognizable departure from the range of normal variation in pigmentation in any of the feathers plucked after irradiation. The bird receiving 2,000 r., however, did show a very striking loss of pigmentation in the great majority of feathers so plucked, although these were normal in structure (see above). The general response is illustrated in Plate 2, where the set of feathers plucked before irradiation can be directly compared with the set plucked 22 months later. None of the latter can be called completely normal in pigmentation, and nearly all of them show gross loss of colour. Details of eight feather generations are given in Table 1.

Particular responses in the serial products of four known follicles. The difficulty of maintaining the identification of follicles over a long period was overcome in the case of four follicles in the bird which had received 2,000 r. Five generations of feathers were produced. The feathers are shown in Plate 4, figs. A–E. The picture as a whole is one of a loss of pigmentation up to 22 months, and then some recovery of it. This is fairly consistent with the results given in Table 1. It is perhaps most clearly seen in feather 3. It appears that at 22 months the pigmented patch in feathers 1 and 2 has moved down the feather by loss of pigmentation at the tip.

TABLE 1

Pigmentary response of feathers to 2,000 r. X-rays

Months after irradiation	*Pigmentation*			*Total number of feathers found*
	Wholly absent	*Much reduced*	*Normal or near normal*	
0	0	0	47	47
0–2+	(pin-feathers)	(pin-feathers)	(pin-feathers)	23
6	7	16	1	24
8	0	9 (immature)	1 (immature)	10
12	5	23	7	38 (3 lost)
19	0	43	0	43
22	11	33	0	44
28	1	35	3	39
Total number of feathers filed after irradiation	24	159	12	195

The micro-anatomical responses of the feather-germ systems mediating the changes in pigmentation

In the birds receiving less than 2,000 r., which showed no pigmentary changes macroscopically, no abnormality of the pigment cell population was found on exhaustive examination apart from the occasional appearance (as in the 2,000-r. bird) of greatly enlarged melanocytes such as the one shown in Plate 1, fig. G. No previous reference has been found to cells of this type. They will be referred to as 'elephant cells'. As can be seen, they are very many times the volume of any normal melanocyte. The melanin in them is normal and not aggregated.

In the bird receiving 2,000 r., four pin-feathers were selected and plucked 9 months after irradiation. The follicles from which they had been taken were kept under constant observation, and they produced the four feathers shown in Plate 4, fig. F (plucked and filed about 3 months later). Two of the original pin-feathers appeared to be white. The follicles from which they came then produced the feathers labelled *A* and *a* in the figure. The other two original pin-feathers appeared to be normally coloured. The follicles from which they came then produced the feathers labelled *B* and *b* in the figure. Pin-feathers *A* (white) and *B* (coloured) were sectioned, and *a* (white) and *b* (coloured) were made into flattened preparations.

When pin-feather *a* (white) was split, it was at once noticed that there was an abnormal number of melanocytes in the mesodermal pulp, which was speckled black when seen under a binocular dissecting microscope. Some of these are shown in Plate 1, fig. F. This is not usually seen, and was not seen when *b* (coloured) was split. Pin-feather *A* (white) was sectioned longitudinally, and a few melanocytes were found— nearly all in the pulp. They were almost completely absent from the ectoderm. Some of those found in the pulp were normal in appearance, particularly low down in the cylinder; some were yellow-elaborating and some were black-elaborating; others were full of melanin in clumps, and had no processes. In no case were they donating melanin to epithelial cells, and they tended to congregate beneath the basement membrane. One of them, black-elaborating, is shown in section in Plate 1, fig. E; their appearance in a flattened preparation is very similar to that of cells in the white portion of a developing black-and-white neck feather in a Light Sussex (Plate 1, fig. H). Here again the melanocytes are not donating melanin, and many of them are in the pulp. The developing barbs (out of focus) have no melanin.

DISCUSSION

From the evidence reported here it seems clear that a dose of rather less than 2,000 r. of X-rays directed upon about 6 sq. cm. of the back of the neck of the Brown Leghorn capon has no appreciable effect on the feathers either as to their shapes or colours. There is neither a serious short-term immediate response nor a long-term lasting effect. With a dose of 2,000 r., however, there is a most marked immediate response and a striking long-term effect in loss of colour.

Short-term effect

The short-term effect can be understood in terms of the response of the feather germ to the injuries resulting from irradiation. The scheme of development which the feather is believed to follow has been described by 'Espinasse (1939), and in terms of this scheme it appears that the reaction of the tissue to the rays is greatest in the collar region of the cylinder. In the collar region the tissue which has not already been keratinized is severely damaged or killed, and growth is stimulated around the margin of the inferior umbilicus. This growth-ring produces a sleeve of ectoderm, whose base is a new collar, and which surrounds a core of mesoderm. This sleeve pushes up within the old cylinder the necrotic tissue of the old collar region around it. The old cylinder is lifted up by the new one growing beneath it, very much as the old feather is lifted up by the new one beneath it during the moult, the difference being that in the moult the old feather is mature (i.e. the inferior umbilicus is closed), whereas in this irradiated material the old cylinder is not even near maturity (the inferior umbilicus therefore still being wide open). As a result the new cylinder tip pushes inside the old cylinder. Later, as the new feather becomes greater in diameter, it does push up the whole of the old feather. There seems little doubt that the immediate short-term structural response of the feather cylinder to X-rays is a special case of its normal reaction to injury, such as cauterization (see 'Espinasse, 1939), and is a local and abnormal moult.

In passing it may perhaps be pointed out that the growth of the smooth-walled cylinder within the old cylinder, and the appearance upon the inner aspect of this new cylinder of pleats forming the barbs, seem to be consonant with the supposition that feather growth is a matter of simple elongation together with pleating, this pleating being apparently due to an innate tendency to produce strips—a tendency which this tissue shows even in culture (Cohen, 1957). It makes any appeal to some form of concrescence quite unnecessary.

Long-term effect

It is far less easy to understand the long-term consequences of irradiation in feather-germs. These effects seem to be almost entirely restricted to changes in the colours of the subsequent feathers. In no case was any significant irregularity in shape found. Any effect of the X-rays on the ectodermal epithelium must be of a most restricted character: it must somehow forbid the acceptance of melanin from melanocytes and do nothing else detectable at all. The melanocytes present in developing feathers which are going to be white are, in this irradiated material, almost entirely excluded from the ectoderm, and it may perhaps be that the basement membrane is somehow changed so that the melanocytes cannot penetrate it. In the white feather parts of Light Sussex fowls, too, the melanocytes, while present, are not almost exclusively in the ectodermal walls of the cylinder, as they are in the black parts, but largely in the mesoderm, and they all (or nearly all) fail to donate melanin, which is often aggregated as in 'mulberry-cells'. How they are excluded from the ectoderm in the white feather parts is not known. It may be that irradiation of Brown Leghorn skin brings about a state of affairs resembling that of the white parts of a Light Sussex feather.

The only piece of evidence, and this is very slight indeed, that seems to suggest that the melanoblast or melanocyte population has been affected directly by the X-rays is that occasionally a very greatly enlarged melanocyte was noticed in the irradiated material (Plate 1, fig. G).

Most puzzling of all, however, is the fact that a number of feathers were found which had coloured tips while the rest of the vane was nearly or quite colourless. This state of affairs was repeated to some extent in series of feathers from one germ. The repetition, if it is real, is not very exact, and the colour varies in quantity (Plate 4).

It is hoped that further work, using tissue culture methods, may add to our knowledge of this matter. Transference of melanocytes or melanoblasts from feather-germs which are white because they have been irradiated may perhaps show whether they have themselves been affected or not. It might be found that they are able to pigment ectodermal cells other than those in the irradiated germ, either *in vitro* or when transferred to other living birds. Should this turn out to be the case then it would seem that the X-rays must be acting upon something other than the melanoblasts or melanocytes, and must be affecting their environment.

SUMMARY

1. Doses of up to 1,000 r. of X-rays administered to a patch on the back of the neck of Brown Leghorn capons had no permanent effects on either the structure or the colours of the feathers subsequently produced in the patch.
2. A dose of 2,000 r. was followed by a marked and permanent loss of colour, but no recognizable structural abnormalities, beyond a slight reduction in size, in the feathers subsequently produced in the patch.
3. The immediate response of an actively growing feather-germ to a dose of 2,000 r. was closely comparable with its known response to traumatic treatment such as cauterization. It can be understood as an abnormal and local moult.
4. Feathers growing in the patch after a dose of 2,000 r., if they had colour at all, had it mostly at their distal ends. Series of feathers which were white with coloured tips were produced from identified follicles.
5. The irradiation appears to have affected the physiological situation of the feather-germs in such a way as to forbid the melanocytes, though present and potentially functional, to enter the ectoderm in the normal way and to donate pigment. The melanocytes in these abnormal circumstances fail to develop normal processes. The melanin in them is in an abnormal physical state of aggregation. This condition is also to be seen in the non-donating 'mulberry-cells' and in the coloured elements of some melanomata. This effect is probably due to a change in the ectoderm, but this change must be of a most restricted character since it does not interfere with the capacity of the ectoderm to produce structurally normal feather. Its nature is not yet understood.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the help and advice given by Professor J. S. Mitchell, F.R.S., on whose suggestion the work was originally undertaken, and by Dr. K. W. Beetham and Mr. T. J. Tulley, who undertook the irradiation of the birds in the Radiotherapy Department at the Hull Royal Infirmary.

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EXPLANATION OF PLATES

PLATE 1

FIG. A. Above, three typical melanocytes with black pigment. Lower centre, one melanocyte with yellow pigment. Lower right, melanocyte with both black and yellow pigments. Section of a developing neck feather from a Brown Leghorn capon.

FIG. B. Pigment-laden cells in human melanoma. Section.

FIG. C. Low-power view of part of flattened preparation of a developing neck feather from a Brown Leghorn capon. Black- and yellow-elaborating cells can be seen in 'synapse'.

FIG. D. 'Mulberry cells' in a flattened preparation of a developing neck feather from a Brown Leghorn capon.

FIG. E. Degenerate melanocyte in the mesoderm (to the right) against the basement membrane separating it from the ectoderm (to the left). Longitudinal section of a developing neck feather from a Brown Leghorn capon after 2,000 r. X-rays. The feather was growing colourless.

FIG. F. Degenerate melanocytes in the mesoderm in a flattened preparation of a growing neck feather from a Brown Leghorn capon after 2,000 r. X-rays. The feather was growing colourless.

FIG. G. 'Elephant cell' in a flattened preparation of a developing neck feather from a Brown Leghorn capon after 1,000 r. X-rays. The feather was growing with normal colour.

FIG. H. Degenerate melanocyte in the mesoderm beneath that part of the ectoderm producing white feather in the neck feather of a Light Sussex hen.

PLATE 2

FIG. A. Feathers plucked from a marked patch on the back of the neck of a Brown Leghorn capon. All are normal.

FIG. B. Feathers plucked from the same patch as above 22 months after it had received 2,000 r. X-rays.

PLATE 3

FIG. A. Longitudinal section of the base of the cylinder of a neck feather from a brown Leghorn capon plucked as a pin-feather 24 hours after the area had received 2,000 r. X-rays.

FIG. B. As for Fig. A, but 48 hours after irradiation. The keratinized inner follicle wall has come out with the feather.

FIG. C. Longitudinal section of regenerating feather. The new ectodermal cylinder has formed a cap over the pulp. As above, but 4 days after irradiation.

FIG. D. As above, but 11 days after irradiation.

FIG. E. T.S. of feather as above 3 days after irradiation, near base.

FIG. F. T.S. of the same feather as shown in Fig. E, but further up the cylinder.

PLATE 4

FIGS. A–E. The feathers produced from four identified follicles numbered 1–4. Five generations are shown in the five figures. The times which had elapsed since the irradiation (dose = 2,000 r.) are as follows: fig. A, 10 months; fig. B, 12 months; fig. C, 19 months; fig. D, 22 months; fig. E, 28 months. (All intervals given to the nearest month.)

FIG. F. The four feathers which were produced by the four follicles lettered *A*, *a*, *B*, and *b* after the pin-feathers similarly lettered had been plucked for sectioning and flattening (see text).

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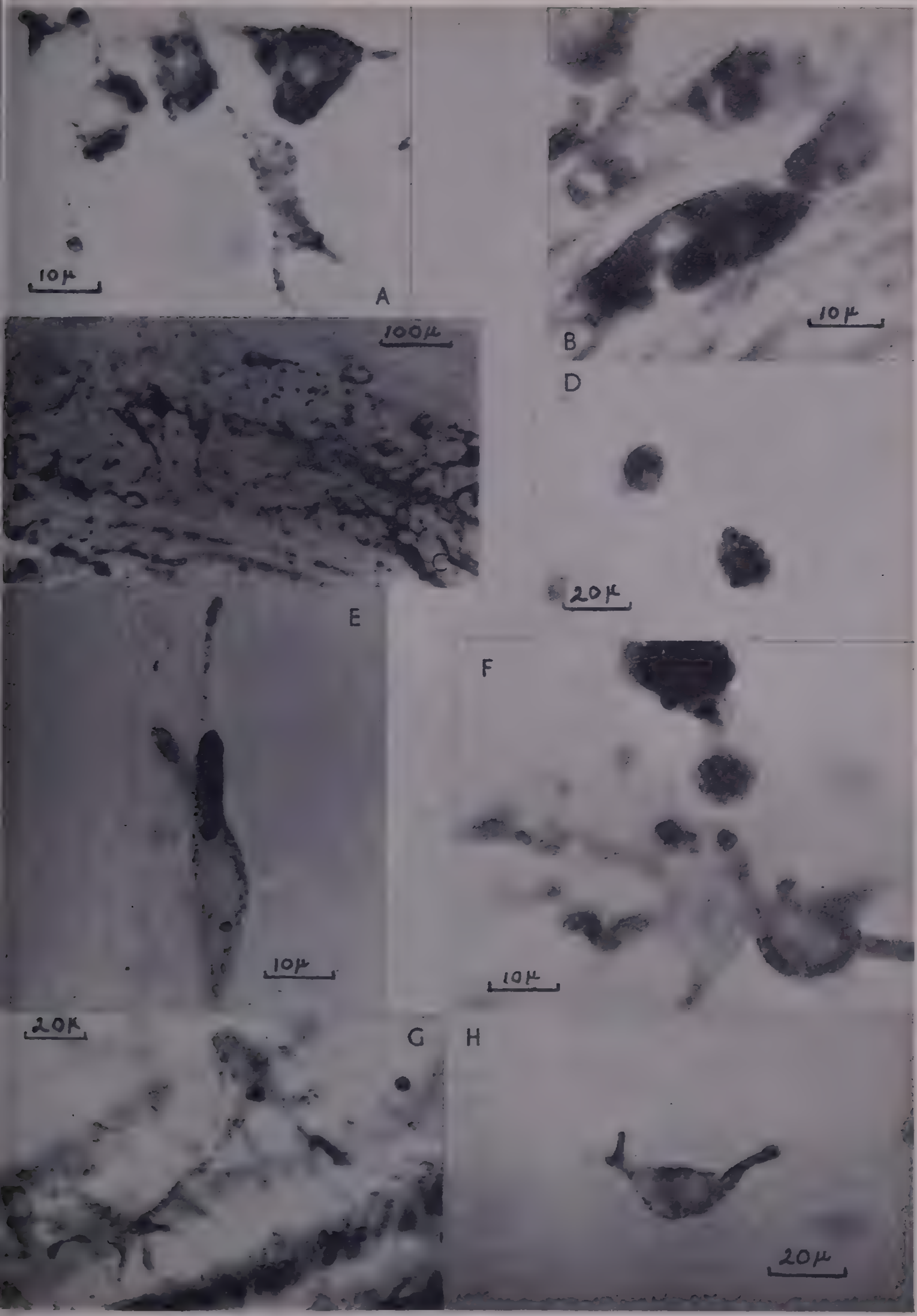

P. G. 'ESPINASSE

Plate 1

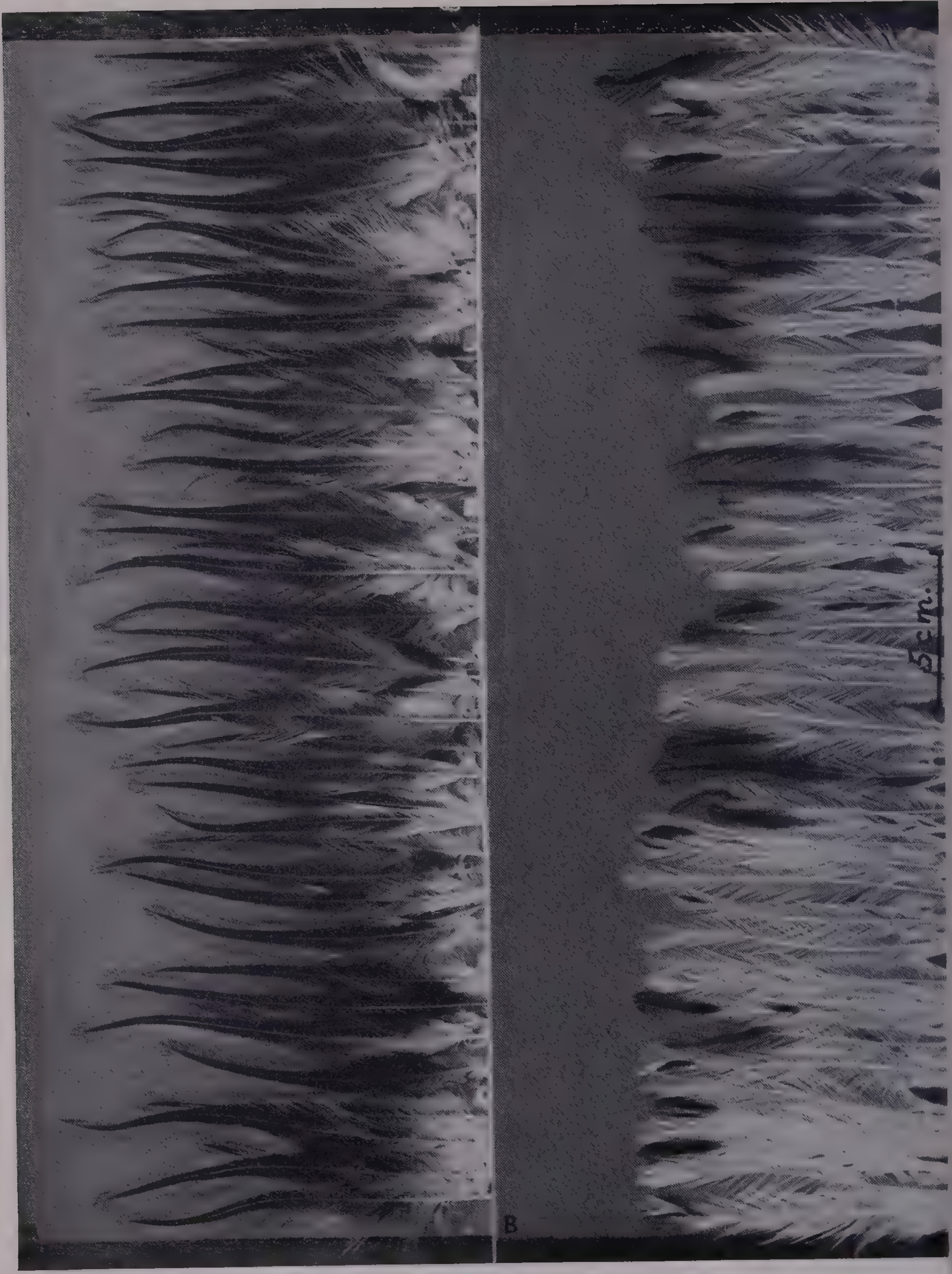

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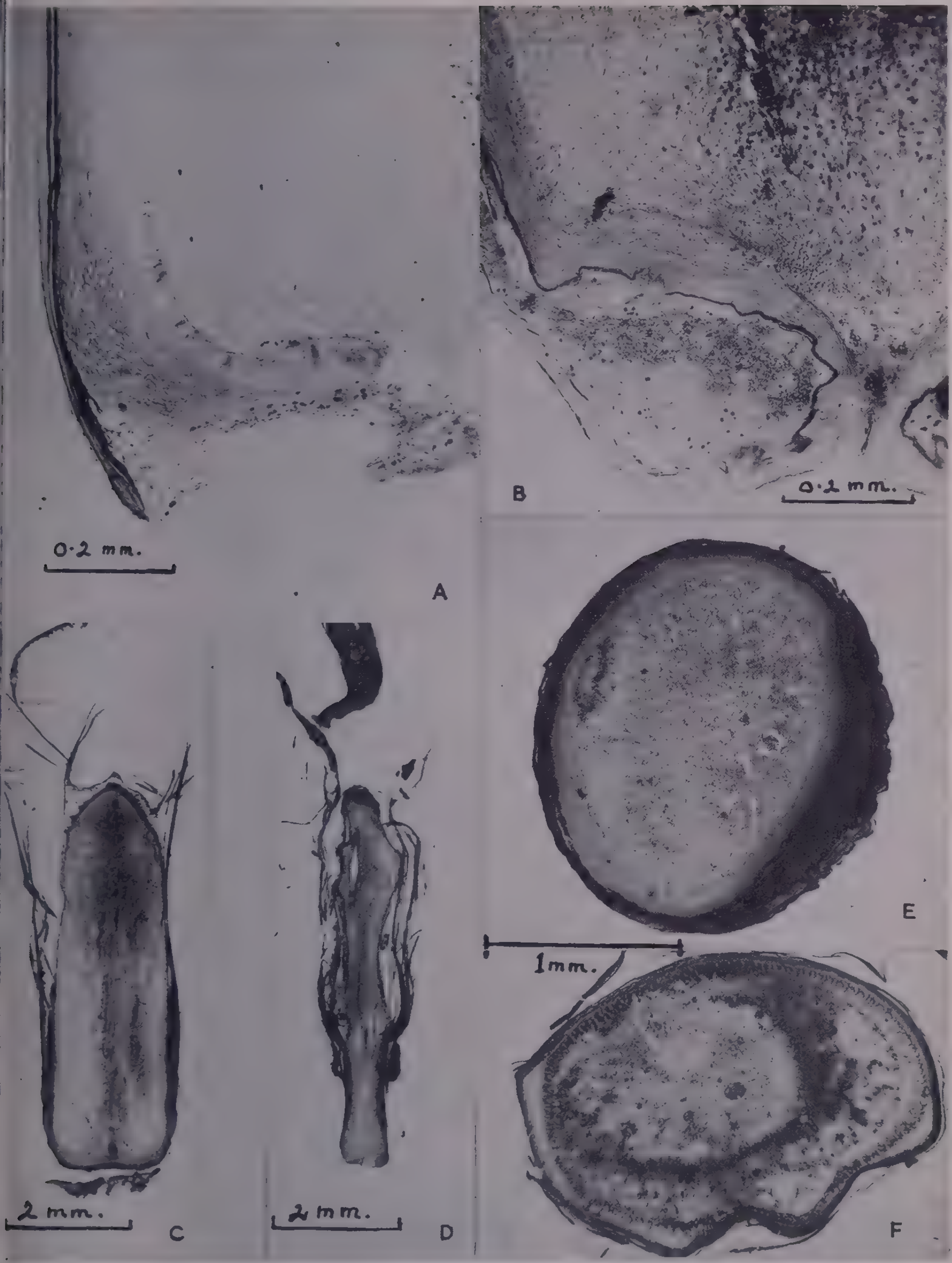
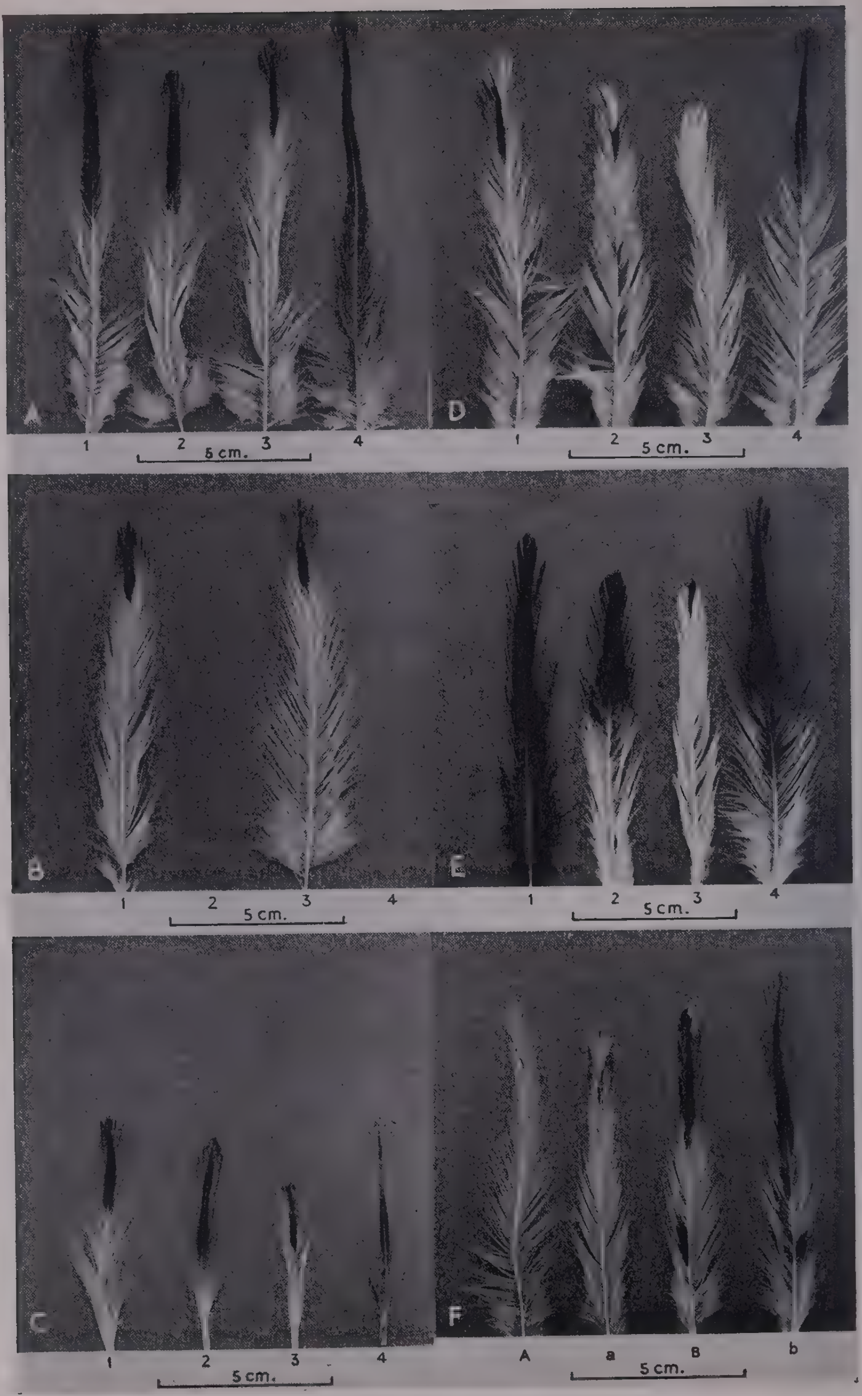
Plate 2



P. G. 'ESPINASSE

Plate 3



P. G. 'ESPINASSE

Plate 4

X-ray Inactivation of Nuclei as a Method for Studying their Function in the Early Development of Fishes

by A. A. NEYFAKH[1]

From the Institute of Animal Morphology of the Academy of Sciences of the U.S.S.R., Moscow

INTRODUCTION

It is generally accepted at present that during cleavage in echinoderms, amphibians, and fishes, the nuclei do not have specific functions in regulating development, their role being at this time restricted to participation in the processes of cleavage (Schechtman & Nishihara, 1955). Eggs devoid of nuclei sometimes begin cleavage which may proceed up to the stage of the late blastula. Extirpation or inactivation of nuclei may be achieved through the separation of the nuclear region of the egg by means of centrifugation (Harvey, 1940); through extirpation of the female nucleus followed by fertilization with sperm inactivated by a heavy dose of radiation (Briggs, Green, & King, 1951); through spontaneous degeneration of the male nucleus during artificial androgenesis (Stauffer, 1945); and by means of other techniques.

Exposure of early cleavage stages in amphibians (Mangold & Peters, 1956; Sanides, 1956) and fishes (Neyfakh, 1956*a*) to heavy doses of ionizing radiation also leads to arrest of development at the late blastula stage. Such arrest at late blastula or early gastrula is observed in many lethal hybrids of echinoderms, amphibians (see the survey of Moore, 1955), and fishes (Korovina, 1939). This syndrome has acquired the special name of 'arrested blastula'.

One may say that the conditions necessary for the development of the egg from its fertilization up to the 'arrested blastula' are already created during ovogenesis (Mazia, 1952) through the activity of the egg's own nucleus and, perhaps, of the nuclei of follicular cells. Thus, beginning with Boveri (1918), the view developed that in ontogeny the nuclei begin their specific developmental function only just before the onset of gastrulation. This viewpoint was recently supported by the fact that the synthesis of specific proteins of the male parent (detected by the appearance of antigenic properties) may be observed in the hybrids of sea-urchins only after the onset of gastrulation (Harding, Harding, & Perlman, 1954; Harding, Harding & Bamberger, 1955).

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It is, however, hardly possible to identify the manifestation of nuclear activity at the onset of gastrulation with the real beginning of nuclear activity: obviously some time has to elapse between the beginning of the activity and its observed results. The methods of investigation referred to above do not allow one to investigate this point, as the extirpation or inactivation of the nucleus is performed almost at the beginning of development, so that the experimenter cannot obtain information about the processes going on between the stage of treatment and that of observation.

The work presented here is an attempt to use selective damage to the genetical nuclear apparatus by heavy doses of X-radiation to investigate nuclear function in the developing embryo.

The nuclei of eggs and early embryos are known to be more highly radiosensitive than the cytoplasm;[1] in a number of cases this has been quantitatively determined. Androgenetic diploid embryos of *Bombyx mori* develop normally after irradiation of eggs with a dose exceeding 500,000 r. before fertilization (Astaurov, 1947). One α-particle entering the nucleus of the *Habrobracon* egg has in the statistical sense the same effect (63 per cent. mortality) as 2×10^7 α-particles entering the cytoplasm; when evaluated in equal volumes this corresponds to a nuclear sensitivity 3,000 times higher than that of the cytoplasm (Borstel & Rogers, 1958). The cytoplasmic sensitivity of fish eggs becomes noticeable after irradiation with doses of the order of 60–80 thousands of röntgens (Neyfakh, 1956*b*), while radiation-induced damage in the nucleus, manifested in decreased embryo survival, becomes evident after treatment with a dose as small as 100 r. Chromosome aberration can be found after a still smaller dose, namely, 25 r. In some cases one may suppose that the damage to the nucleus is determined indirectly, through the action of irradiated cytoplasm. But even if this effect plays some part, it does not affect the main conclusion that the disturbance of development and activity of the embryo is determined by the radiation effect upon the nucleus (Astaurov, 1958).

It seems, therefore, practicable to find a dose-range within which the radiation destruction of the nucleus will be practically complete, whereas that of the cytoplasm will not yet be significant. Under these conditions the radiation effect may be compared to the simultaneous extirpation of all the nuclei from the embryo. Embryonic development after such a procedure can proceed only up to the time determined by the preceding nuclear activity and may show itself as a temporary ability of the cytoplasm to undergo independent development.

MATERIAL AND METHOD

The work was carried out on embryos of the pond-loach (groundling), *Misgurnus fossilis* L. This species has until recently been undeservedly little used in embryological and radio-embryological studies. The possibility of working the year round, the simplicity with which the adult fish may be kept, the ease with which mature gametes may be obtained, their abundance, and the tolerance of the eggs when incubated, permit one to class the loach with such embryological material as the frog, chick, or sea-urchin.

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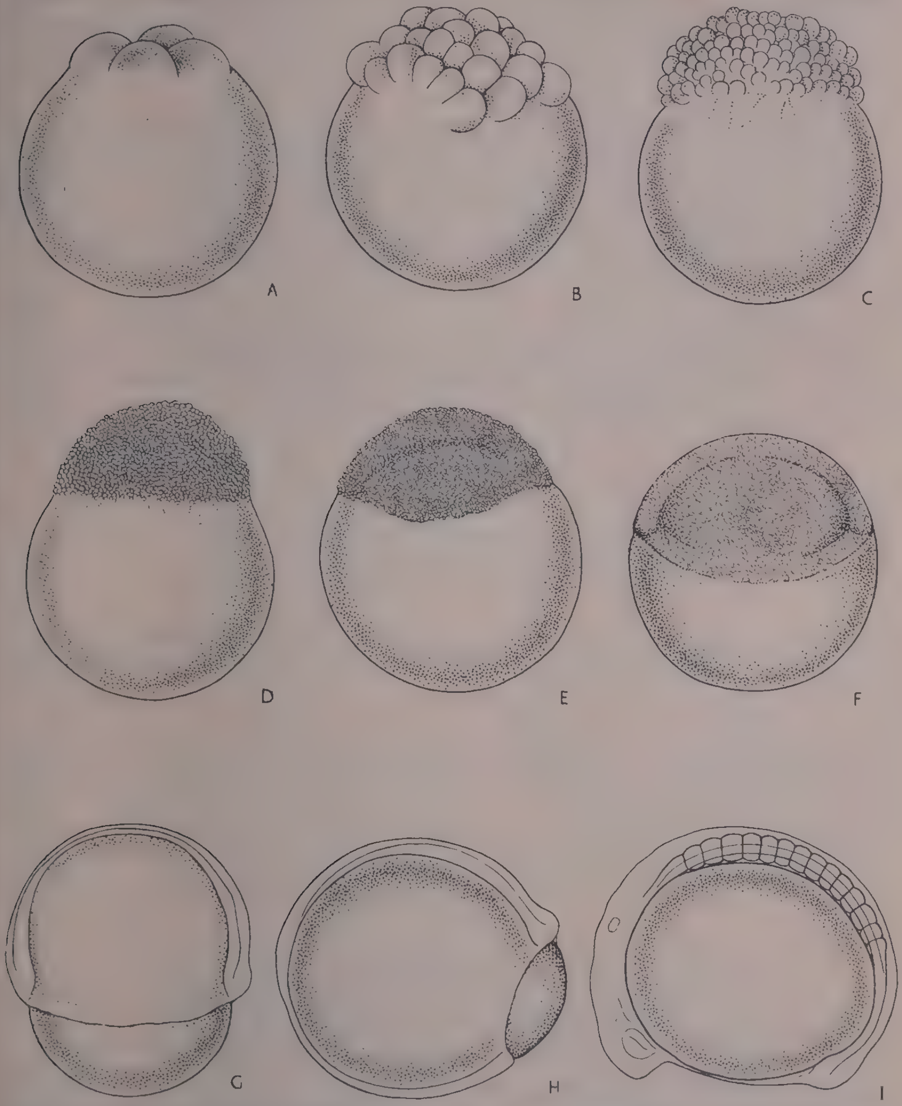
[1] This expression, and analogous ones used below, is intended to mean not only that the nucleus is more injured than the cytoplasm, but also that nuclear damage is more often fatal to the cell than equivalent damage to the cytoplasm.

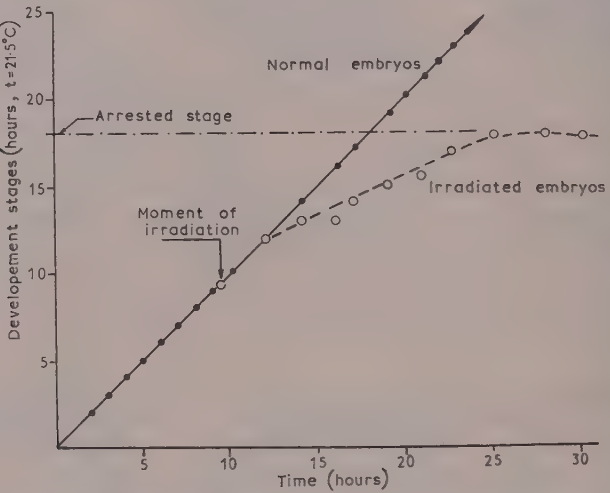


Text-fig. 1. Stages of normal development of the loach. Temperature 21·5°. A, 2 hours, 4 blastomeres; B, 4 hours, 32 blastomeres; C, 5 hours, morula; D, 7 hours, mid-blastula; E, 9 hours, late blastula; F, 11 hours, early gastrula; G, 15 hours, mid-gastrula; H, 18 hours, end of gastrulation; I, 28 hours, 13 somites.

Mature gonad products were obtained 30–36 hours (temperature 18° C.) after injection of a chorionic gonadotropic hormone preparation (200 rat units, preparation of the Institute of Experimental Endocrinology, Moscow). Artificially inseminated eggs with a fertilization rate of 70–98 per cent. were incubated in Petri dishes, up to 250 eggs per dish of 9 cm. in diameter at a temperature of 21–22° C., which is the upper optimal limit.

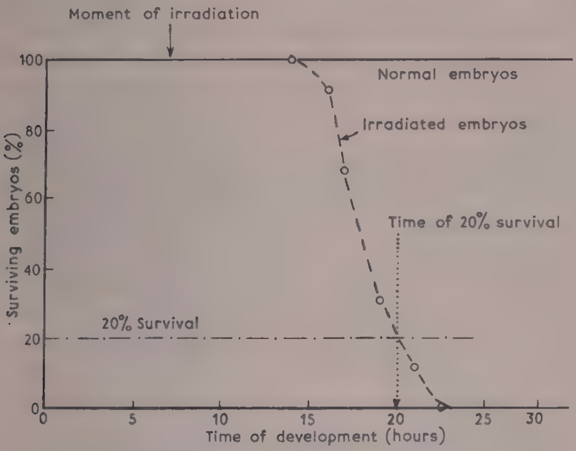
Developmental stages are shown in part in Text-fig. 1. Each stage is referred to by the number of hours of development required to reach it at 21·5° C. Even if the actual temperature of incubation is lower, and development consequently slower, the same method of referring to the stages is used. Thus the early gastrula stage '13 hours', begins after 13, 19, and 26 hours at temperatures of 21·5°, 18°, and 13°, respectively.

Irradiation was carried out in a small volume of water (closely packed eggs were just covered with water) with the X-ray apparatus RUP–1, using 185 kV., 15 mA., no filter, HVL–3 mm. Cu., distance of 75–120 mm., and dose rate of 2–5 kr./min. Exposure time, depending on the dose, was from 1 to 20 minutes.



TEXT-FIG. 2. Method of determination of the maximal developmental stage reached (arrested stage). Irradiation with 20 kr. at the 9½-hour stage. Development is arrested at the 18-hour stage.

As criteria of the radiation effect, the amount of development and the time of survival were chosen. The amount of development was expressed as the maximal stage reached ('arrested stage', designated by hours of normal development at 21·5° C.). The later development of irradiated embryos always proceeds slowly and the actual time taken was disregarded. Text-fig. 2 shows the time course of development for normal and irradiated embryos and the way in which the 'arrested stage' is determined. Individual fluctuations in development are small when heavy doses are applied. The error in assigning the developmental stage to normal embryos does not exceed ½–1 hour, but is somewhat more in irradiated ones. Determination of the developmental stage in malformed embryos at post-gastrulation stages is to a certain degree subjective. When comparing two embryos, however, the more developed is readily distinguished from the earlier



TEXT-FIG. 3. Method of determination of the time of 20 per cent. survival. Irradiation with 20 kr. at the 6½-hour stage. Eighty per cent. mortality had occurred by 20 hours.

arrested. Survival data, supplementing the data on development, provide more precise quantitative expression than stage determination for malformed embryos.

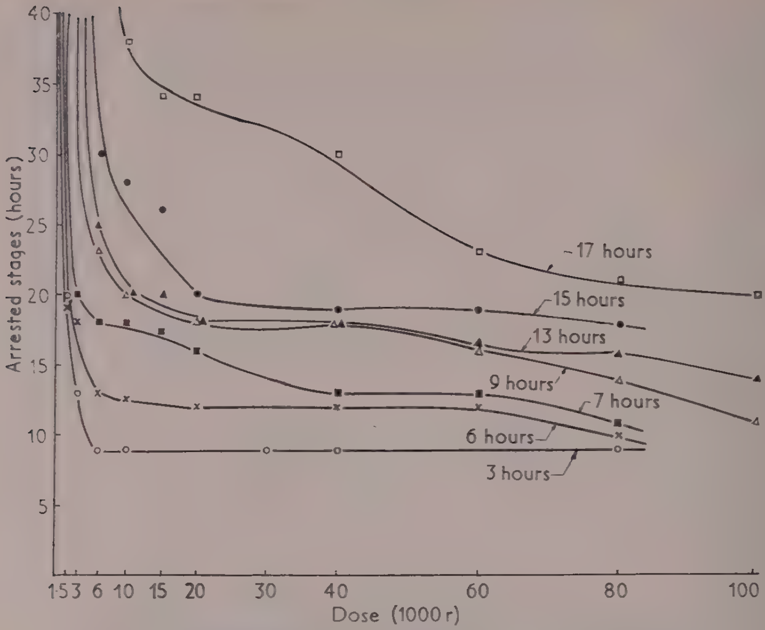
As the survival criterion, the time in hours at standard temperature when an 80 per cent. mortality is reached was chosen (Text-fig. 3). This time differed little (1–2 hours) from that of total mortality, but it could be determined more precisely. Counting of dead embryos and assessment of stage were carried out every 1–2 hours. The error in the determination of the moment of 80 per cent. mortality of the embryos is at most half this interval.

RESULTS

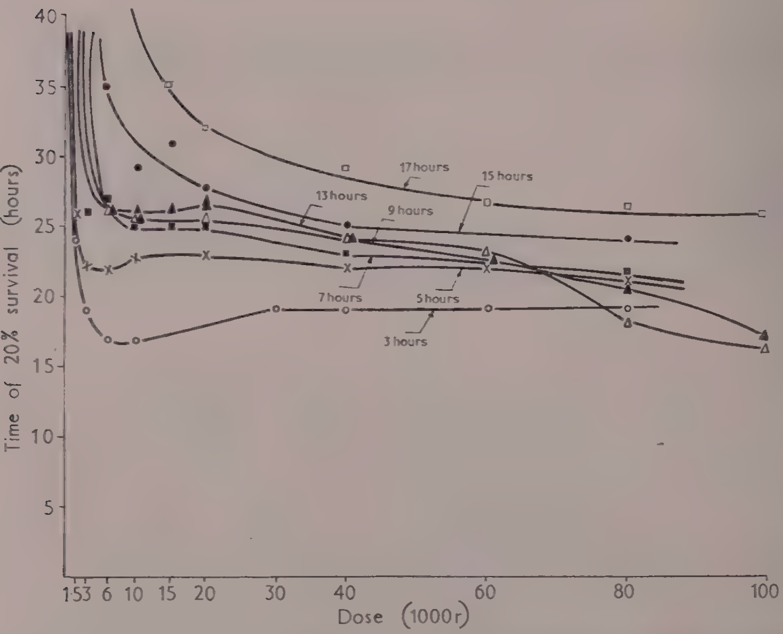
Dose dependence at various developmental stages

In order to select radiation doses meeting the aim of this work, the eggs were exposed to irradiation with doses ranging from 1·5 to 100 kr. at different developmental stages. The results expressed in the maximal stage reached ('arrested stage') and in the 20 per cent. survival time are presented in Text-figs. 4 and 5.

The curves representing the dependence of development on dose are more or less similar with different irradiated stages. The steep exponential drop with small doses is succeeded by complete, or almost complete, parallelism to the abscissa. With very heavy doses the slope of the curves somewhat increases again. The dose at which the inflexion of the curve takes place is less when



TEXT-FIG. 4. Dependence of the arrested stage on the irradiation dose.



TEXT-FIG. 5. Dependence of the time of survival of 20 per cent. of embryos on the irradiation dose.

irradiating earlier (at the 3-hour stage, 6 kr.; at the 5-hour stage, 10 kr.) than when irradiating later (at the 13- and 15-hour stages, 20 kr.). When irradiating at the 7-hour stage the inflexion is less marked and a certain slope is retained throughout the curve. When irradiating at the 17-hour stage there is no plateau though the slope is small with large doses.

Survival curves have the same pattern in general. Irradiation at the earliest time (up to 3 hours) is, however, an exception: here a paradoxical effect is observed, namely, a slightly increased survival time with heavier doses. This is explained by some delay in cleavage, so that the more strongly irradiated embryos attain the stage when death occurs later than those irradiated with lower doses. This cleavage delay is marked in sea-urchins (Henshaw, 1940) and other invertebrates, less marked in amphibians (Kheissina, 1956), and slight in fishes.

The strong dependence on dose seen in the left part of the curves can only be explained by damage to the genetical structure of the nucleus. The plateau formation within the dose range of 6–20 to 60 kr. means that with these doses radio-sensitive structures are maximally damaged. Thus 20 kr. may serve as the lower dose limit meeting the requirements of the present work. The upper limit cannot be defined precisely enough, but 60 kr. is probably the dose at which cytoplasmic effects become significant at later developmental stages. At the end of gastrulation (17 hours) application of heavy doses cannot give precise results comparable to those obtained at earlier stages because of the appearance of some dependence on dose within the range chosen.

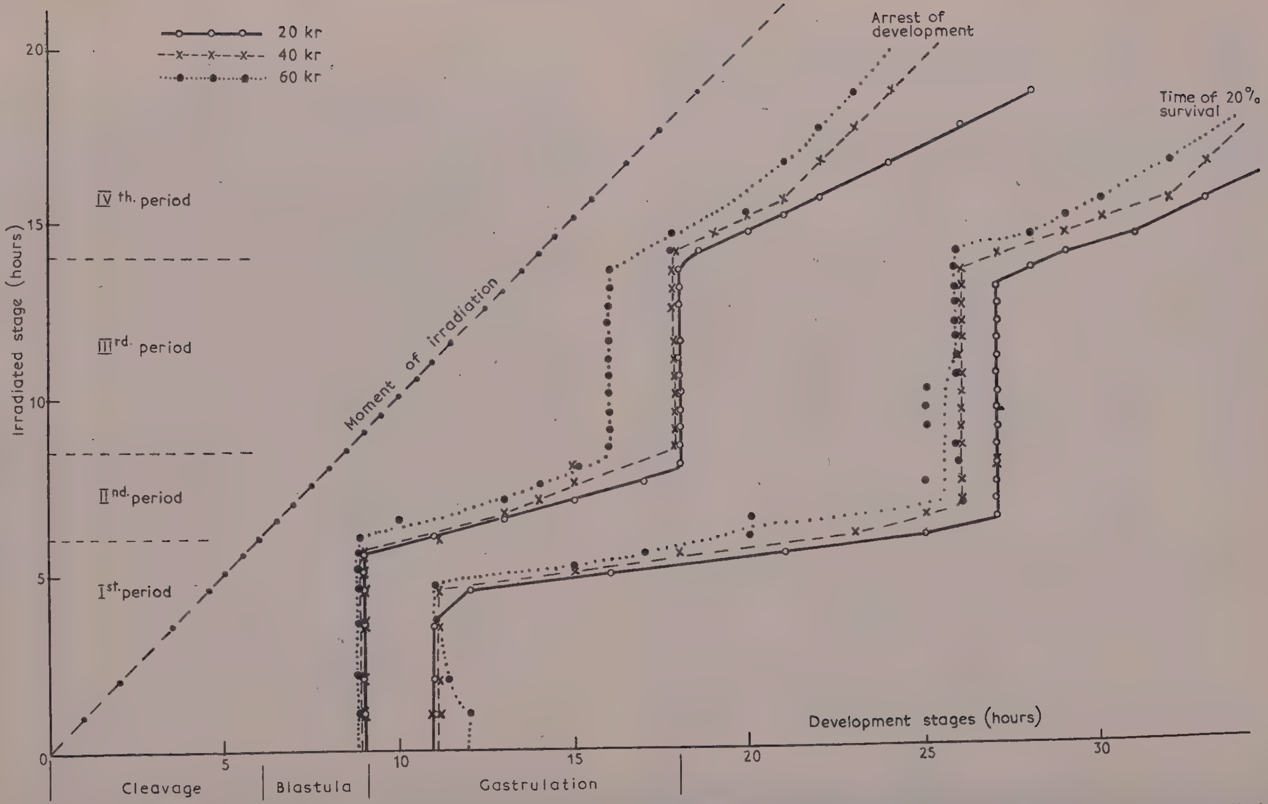
Dependence of development and survival on the stage of exposure to irradiation

Data on irradiation at different developmental stages are set out in Text-figs. 6 and 7. The later the stage exposed to irradiation, the higher (along the ordinate) lie the points corresponding to the moment of irradiation, arrest of development, and 20 per cent. survival. The curve connecting the points of arrest of development shows the dependence of the stage of developmental arrest on the stage of irradiation: the curve connecting the points representing 20 per cent. survival similarly shows the dependence of survival on stage of irradiation.

Text-fig. 6 shows the results of one experiment. The different curves correspond to different irradiation doses. The lower part of the curves, concerned with the first few hours of development, has been described in more detail elsewhere (Neyfakh & Rott, 1958) and will be considered here only briefly. The various periods into which the curves fall were carefully studied in many experiments, and in all cases the results did not differ essentially. Text-fig. 7 shows some curves obtained in analogous experiments to demonstrate the reproducibility of the pattern of the curves.

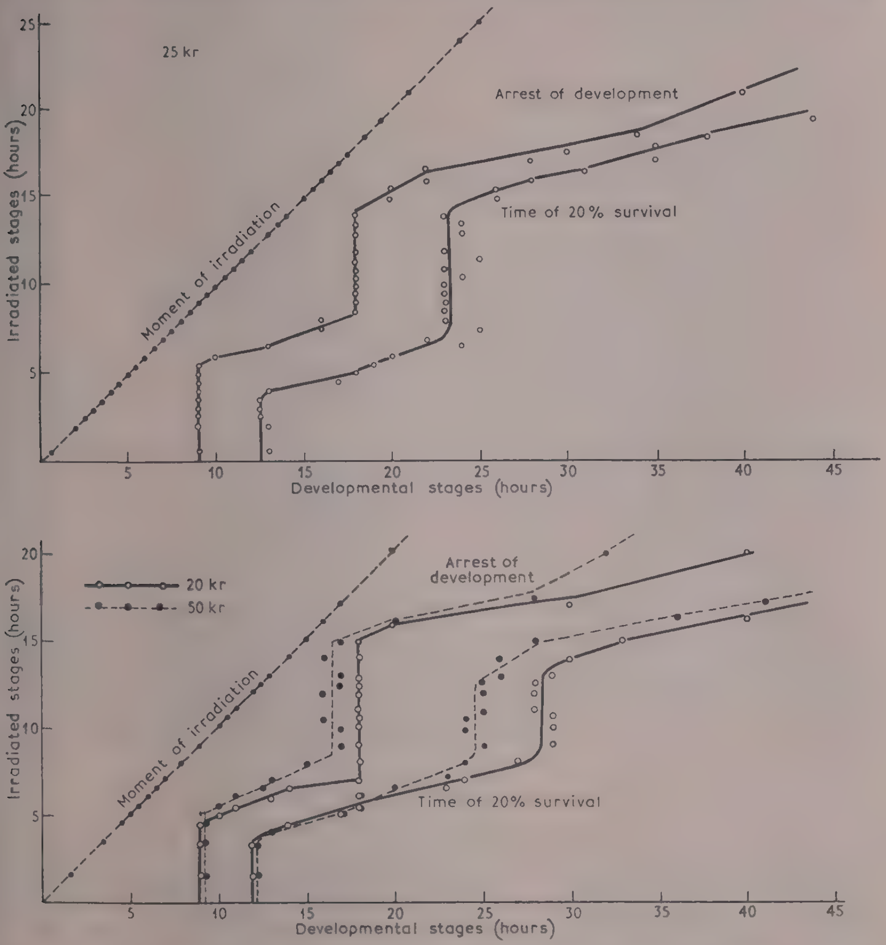
The curve relating development and stage of irradiation may be divided into four periods.

The first period covers irradiation from the beginning of development to the stage of the early large-celled blastula (from 0 to 6 hours). The development of all



TEXT-FIG. 6. Effect of high irradiation doses (20, 40, and 60 kr.) on different developmental stages. Each point on the line labelled 'moment of irradiation' represents the irradiation of one series of embryos. The stage at irradiation may be determined by the projection of this point either on the ordinate or on the abscissa. The development of the irradiated embryo proceeds from the moment of irradiation up to the arrested stage and the amount of development may be determined as the horizontal distance between the 'moment of irradiation' and the corresponding point on one of the curves labelled 'arrest of development'. The different curves correspond to different dosages. The embryo arrested in its development survives for a time. Eighty per cent. of the embryos are dead at the time (the

the embryos exposed to irradiation during this period is arrested at the stage of late blastula ('arrested blastula'). No differences were found between doses within the range from 20 to 60 kr., as in the experiment of Text-fig. 6, or from 6 to 80 kr. in other experiments. There is no dependence on the stage of irradiation within this period.



TEXT-FIG. 7. Further experiments on the effect of high radiation doses on different developmental stages. For explanation of the method of plotting see legend to Text-fig. 6.

The second period covers irradiation from the early to the late blastula (from 6 to 8½ hours). Its range may vary in different experiments by half an hour of standard development. The arrest of development during this period, unlike the preceding one, depends sharply on the stage of irradiation. An embryo exposed to radiation 1 hour later than another halts its development at a stage equivalent to 2–4 hours later. During this period some dependence on dose is observed (cf. the curve for the 7th hour, Text-fig. 4), though as a whole the curves for all the three doses of Text-fig. 6 (20, 40, 60 kr.) closely follow each other.

The third period covers irradiation from the late blastula to the mid-gastrula (from $8\frac{1}{2}$ to 14–15 hours). As in the first period, no dependence on stage of irradiation was observed within this period. But the anlage of the axial organs, normally appearing by this time, is lacking in irradiated embryos. Some dependence on dose during this period is manifested in the fact that after 20 to 40 kr. the yolk becomes entirely covered, whereas after 60 kr. development stops at the 16-hour stage, before the yolk is covered.

The fourth period starts with the mid-gastrula. The criterion of the beginning of this period, a small bulge produced by the first formation of axial organs, cannot unfortunately be defined with precision. The effects of irradiation of later stages were followed in the experiment of Text-fig. 6 beyond the end of gastrulation (to the 20-hour stage). During this period a marked dependence on stage of irradiation was again observed. Some dependence on the dose also begins to appear, so that the designation of a stage as the maximal one reached begins to lose its significance; though with all the doses applied (20–60 kr.), through the whole period, there is no doubt of the objective dependence of development on the stage of irradiation. Development after irradiating at the stage of the first somites, though it cannot be estimated precisely enough, proceeds up to hatching, when the death of the majority of embryos prevents further observations.

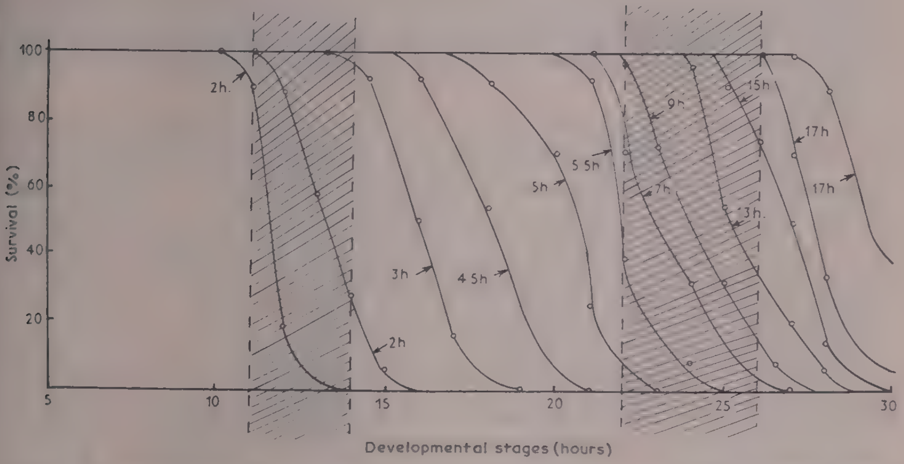
Survival curves, in general, follow rather closely the developmental curves. As with the latter, four periods may be distinguished, of which the first and third are characterized by independence of stage of irradiation, whereas the second and fourth are characterized by strong dependence. A dependence on dose is observed to a small degree in the third period, and to a greater degree in the fourth period.

The essential peculiarity of the 20 per cent. survival curve is the fact that all its transitions from one period to the next occur at an irradiation stage $1–1\frac{1}{2}$ hours younger than those of the curve of arrest of development. This independence shows that the processes concerned in arrest of development and in mortality, though closely related, are not quite identical.

Mortality dynamics

Increased mortality of the embryos at 11–14 hours of development (at standard temperature) after irradiation in the first period, and at 22–26 hours after irradiation in the second period, may suggest that there are non-specific 'critical stages' at these times. Such an idea can be tested by analysis of the curves showing the dependence of mortality on time. Mortality of the embryo in 'critical stages', if such exist with respect to radiation, must increase with time relatively independently of the stage of irradiation and of the dose, and must be determined only by the developmental stage of the embryo.

Text-fig. 8 shows mortality curves obtained when varying the stage of irradiation with doses of 20–40 kr. From the 11th hour of development a mortality may be achieved which begins and ends at almost any moment of development. Thereby, the curves which are completely included within the suggested 'critical stages' do not differ from others which either start in these periods, end in them, or lie beyond them.



TEXT-FIG. 8. Dependence of embryo mortality on the developmental stage reached after irradiating at different periods and with doses of 20 and 40 kr. Developmental stages at the moment of irradiation (in hours) are given for each curve. The curves have been artificially selected from various experiments with the sole intention of showing that death of the embryos may be observed at very different ages. The shaded areas are the supposed 'critical stages' (see text); no especially sharp fall of the curves occurs during them.

DISCUSSION

Effect of heavy doses of ionizing radiation

Analysis of the dependence of radiation effect on dose (Text-figs. 4 and 5) show that the early fish embryo represents, with respect to radiation, a system of two components with different sensitivity or different role. Damage to the first component (the radio-sensitive one) occurs at doses of the order of 6–20 kr., whereas damage to the second component (the radio-resistant one) begins at doses of the order of 60–100 kr. All the data available in the literature suggest that it is the cell nuclei that are likely to be the radio-sensitive component and the cytoplasm the radio-resistant one. After irradiation of the eggs or early embryos with heavy doses arrest of development takes place at the late blastula stage, which is typical for nuclear injuries. Only by increasing the dose up to 80–100 kr. can one obtain an earlier or even immediate effect (arrest of development, death) caused by injury to the cytoplasm (Neyfakh, 1956*b*). Periodical increases of radio-sensitivity in the course of cleavage precisely coincide with the rhythm of the cleavage divisions (Neyfakh, 1956*a*) and correspond to telophase (Belyayeva & Pokrovskaya, 1959). It is obvious that here, as in other material (Luther, 1938; Henshaw, 1940) the change in radio-sensitivity is determined by the changes in chromosome structure; the chromosomes are, therefore, the primary objects of damage.

The increase and decrease of the mortality rate of embryos when irradiated at different phases of the mitotic cycle coincide with an increase and decrease of chromosome aberration frequency, which directly proves the injury to the genetic apparatus.

When applying larger radiation doses, of the order employed in the present work, the injuries to the nucleus become even more profound. Karyokinesis is depressed (though cytokinesis is retained in early cleavage), and atypical and pycnotic forms of nuclei appear. A Feulgen-positive substance is found outside the nuclei and even outside the cells (Belyayeva & Pokrovskaya, 1958). An analogous appearance of Feulgen-negative nuclei was described in amphibians (Kheissina, 1956) and in *Habrobracon* (Borstel, 1955).

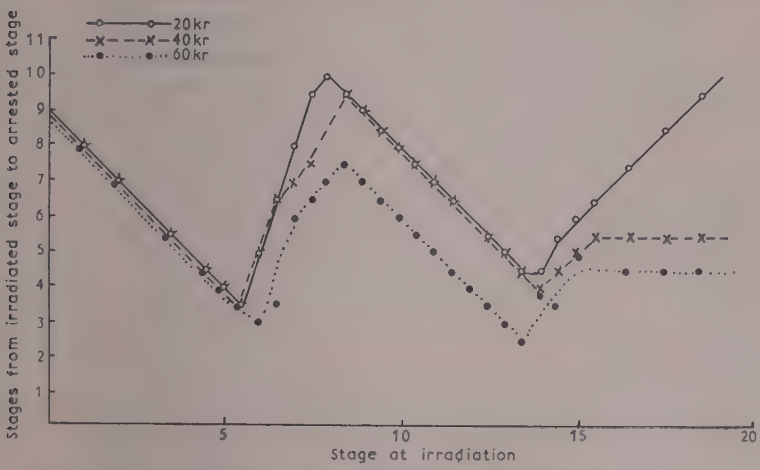
Radiation injury of the cytoplasm may be ascribed to the doses of 60–100 kr. only tentatively. The precise form of this effect, its dependence on dose and on developmental stage, have been studied but little. It would therefore be more correct to confine oneself to the statement that up to a dose of 60 kr. the primary radiation injury to the cytoplasm is of a restricted character, usually undetectable in development.

Analysis of the dose-effect curves permit us to state that with doses of 6–20 kr., in which range there is a complete or almost complete independence of effect on dose, the injury of the radio-sensitive component is the maximum possible for ionizing radiation. So far as it is development and survival which serve as the criteria of the radiation effect, it may be rigorously stated that with the above doses the activity of the radio-sensitive component in the regulation of development and viability is completely inhibited.

An important question arises as to how soon the inactivation of the nucleus takes place after irradiation. If the time required for inactivation of the nucleus depends to some extent on the radiation dose (within the range of heavy doses used) one may expect that during the period of nuclear activity a certain dependence on dose will be observed, due to the fact that a smaller dose (e.g. 20 kr.) inactivates the nucleus somewhat later than a heavier one (e.g. 40 kr.). Text-figs. 6 and 4 (for the 7th hour) show indeed that such a dependence exists. Since, however, this dose-dependence, even with a wide dose range, does not exceed 2–2½ hours, which corresponds to 40–50 minutes of nuclear function during this period, it appears that the time required for inactivation of the nucleus after irradiation is not large and indeed hardly significantly exceeds the error of the method, which has been evaluated as ½–1 hour.

The role of the nucleus in the effect of radiation on development

Inactivation of the nuclear genetic apparatus does not lead to the immediate arrest of development. For some time after irradiation the development proceeds at the normal tempo, then this tempo slows down and at last development is arrested (cf. Text-fig. 2). Depending on the irradiation stage, the embryo can pass through stages corresponding to 3–10 hours of normal development. This is possible because development itself is realized by radio-resistant cytoplasmic structures, which may exist independently for some time. Nevertheless, the connexion with the nucleus is here much closer than, for example, in *Acetabularia* or amoeba which may exist without the nucleus for a long time, retaining the power of protein, and, in *Acetabularia*, even of ribonucleic acid, synthesis (Brachet, 1952; Chantrenne, 1958).



TEXT-FIG. 9. Duration of subsequent development (expressed in stages of normal development) after irradiation, depending on the stage of exposure. Plotted from the same material as Text-fig. 6.

The nucleus may be regarded from two aspects: as the site of the formation of non-specific substances necessary for the realization of the developmental processes in the cytoplasm, and as the source of specific hereditary information by means of which the genotype controls the development of phenotypical character (Brachet, 1952; Mazia, 1952; Stern, 1955; Chantrenne, 1958). One may conceive that the nucleus excretes a limited amount of substances consumed in the course of development, or that a certain structural organization arises which is able to ensure only the immediately succeeding developmental processes. In both cases the fate of the embryo after irradiation is determined by the 'stock' present in the cytoplasm at the moment of irradiation.

Analysing the prolongation of development after irradiation at different stages

(Text-fig. 9) one may see that the amount of this ‘stock’ is different at different moments of development. This leads to the conclusion that the nucleus functions either periodically or with varying intensity. Analysis of the data presented in Text-figs. 6 and 7 suggests a solution of the problem as to which it is. Let us analyse the first and third developmental periods (0–6 and 8½–14 hours) in which the stage of arrest of development is independent of the stage of irradiation. The cytoplasm of the embryo irradiated within such a period is able to develop without the nucleus up to the stage of arrest. This ability of the cytoplasm is not altered during the whole period; the later the nucleus is inactivated by irradiation within this period the smaller is the ‘stock’ remaining within the cytoplasm.

This implies that during the first and third periods the nucleus does not increase the ability of the cytoplasm to undergo independent development, does not synthesize the hypothetical substances or structures which are consumed in the course of the development. In other words, in the course of normal ontogeny development is realized during these periods without the constant control of the nucleus on account of nuclear products produced before the onset of the period.

Strictly speaking, one may claim that if irradiation at different moments of a given period leads to arrest of development at one and the same stage, the part of the cell affected by radiation does not during this period influence at all the ability of the rest of the cell to develop. But this certainly does not imply that the nucleus is in a kind of anabiosis during these periods of inactivity. It may participate in various preparative metabolic processes not connected directly with development.

During the second and fourth periods (6–8½ and beyond 14 hours) the ability of the cytoplasm to develop independently increases. Inactivation of the nucleus at every later moment (within the period) finds the embryo in a different state: its cytoplasm contains a larger amount of nuclear ‘information’ about further development than at the preceding irradiation. Strictly speaking, one may assert that if the irradiation at successively later moments of a given period leads to arrest at ever later developmental stages, the structure affected by irradiation does, during this period, significantly affect the ability of the non-damaged part of the cell to develop. Thus, during the second and fourth periods the nucleus actively produces a ‘stock’ of substances or structure which is necessary for further developmental processes.

Possibilities of other interpretations

One might try to interpret the data obtained in terms of radio-sensitivity. Radio-sensitivity depends upon the degree of injury to cellular structures and on the role of these structures in the activity of the cell. The changes of cell sensitivity which depend on the phase of mitotic cycle, and the changes in embryo-sensitivity which depend on the ratio of cells undergoing division, cannot play any role in these experiments, in which the injury is so large that dose dependence no longer exists. The changes in the embryo’s response to irradiation

(Text-fig. 9) must, therefore, be explained by the role of structures affected by irradiation, i.e. of the nuclei, in development.

In many experiments on the effect of injurious agents, of high temperature in particular, arrest of development and increase of mortality were observed at stages close to those characteristic of heavy radiation (late blastula and end of gastrulation; cf. the survey of Trifonova, 1949). The possibility arises therefore of the non-specific character of the phenomena observed. The data available in the literature supplemented by the observation of the author on the effect of high temperature (33° C. for 1 hour) on loach development led to the following conclusions:

1. Arrest of development at the late blastula takes place only in some embryos, whereas others may either be arrested before this stage or may develop further. This is more striking in the case of mortality which, to a significant degree, takes place at the moment of treatment or immediately after it, and then at all succeeding stages with a relative increase at the late blastula and early gastrula.

2. The effect of high temperature significantly injures the nuclear apparatus of embryonic cells. This is shown by the differential temperature sensitivity of different phases of the maturation divisions and of cleavage detected in sea-urchins, silkworms (Ostryakova-Varshaver, 1952), and loach (Vakhrameyeva & Neyfakh, 1959). Thereby nuclear disturbances arise (Lieder, 1954) which are very similar to chromosome aberrations of radiation origin. Even ultrasound leads primarily to disturbance of mitosis (Bonhomme & Pourhadi, 1957).

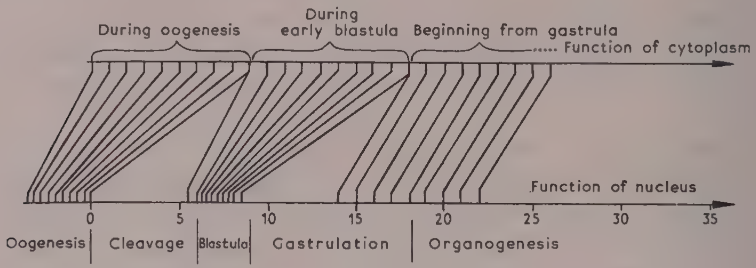
Thus one may suppose that the similarity between such injurious agents as high temperature and heavy doses of ionizing radiation is due to the fact that these agents all injure the nucleus to a significant degree. Denaturation injuries of other parts of the cell besides the nucleus complicate the injury pattern induced by high temperatures, so that its similarity to the effect of radiation is only relative.

The role of the nucleus in the early development of the loach

Inactivation of the genetic apparatus of nuclei at different developmental stages allows one to determine the periods of active function of the nucleus. A scheme of the time and duration of periods of nuclear functioning and the stages determined by these periods is shown, for the early development of loach, in Text-fig. 10.

The first developmental period—from fertilization to late blastula—is determined during ovogenesis by the activity of the egg nucleus and perhaps by that of the follicular cell nuclei (Mazia, 1952). It is natural that during this period conditions are created which are necessary not merely for cleavage and blastulation: yolk synthesis, membrane formation, &c., are needed for the whole of embryonic development. However, the independent development of the cytoplasm may be realized only up to the stage of the late blastula.

The embryo's nuclei begin their specific function in the early blastula and continue it for $2\frac{1}{2}$–3 hours up to the stage of late blastula. As a result the embryo becomes able to develop from the late blastula up to the end of gastrulation. In the middle of gastrulation a new period of nuclear activity begins, which ensures development at post-gastrula stages.



TEXT-FIG. 10. Scheme of the relations of nucleus to cytoplasm in the early development of the loach. The lower scale gives the hours and stages of normal development. The lines connecting the upper and lower horizontal lines indicate which particular stage of embryonic development is influenced by nuclear activity occurring at a given stage. Above the upper line the period at which the nucleus determines the given developmental stages is shown.

The scheme suggested does not conform to the idea that nuclear activity begins in the late blastula, or to the idea that the further activity of the nucleus is continuous. As mentioned in the introduction, however, such ideas are based on inadequate methods for investigating the very beginning of nuclear function; even such a criterion as ribonucleic acid synthesis in the nucleolus (Brachet, 1952) may be secondary to the processes occurring directly in chromosomes. The idea of a temporal succession of developmental processes beginning in the nucleus and ending in morphological transformations figured in the literature long ago. Thus Voss (1933) separated physiological activity (nuclear function) from morphological activity (cytoplasmic function), suggesting a scheme according to which gastrulation is preceded by a period of nuclear function beginning at the late blastula. It was shown later that the appearance of antigens precedes the morphological stages characterized by these antigenic properties (Clayton, 1953). It seems that the supposition that genetical nuclear function begins at an even earlier period does not contradict these ideas.

Several investigations have linked together an increased metabolism with the onset of gastrulation. Nuclear defects in lethal hybrids result in disturbance of normal metabolism precisely at this stage: the cessation of deoxyribonucleic acid synthesis (Chen, 1954; Gregg & Løvtrup, 1955; Løvtrup & Werdinius, 1957), the cessation of the increase in oxygen consumption, and perhaps other phenomena. This does not contradict the scheme suggested, as the onset of nuclear function may be overlooked against the background of the metabolic transformations which start later. Very interesting, however, seem the data recently obtained on protein metabolism in sea-urchin embryos and on ribonuclease activity in early amphibian embryos. Synthesis of structural proteins, which is connected with the preliminary breakdown of reserve yolk proteins in the eggs of sea-urchins, has three maxima: at the early and late blastula, and at the mid-gastrula (Kavanau, 1954). In frogs, ribonuclease activity greatly increases at the early blastula and then at the mid-gastrula (Finamore, 1955). Though we cannot expect to find a complete analogy between the activity phases of nuclear function of echinoderms, amphibians, and fishes, it is evident that at the early blastula and mid-gastrula, just when an increased nuclear activity seems to be found in fishes, important metabolic transformations take place in echinoderms and amphibians.

It has been shown (Neyfakh & Rott, 1958) that in the loach and sturgeon (*Acipenser stellatus*) the synchrony of cleavage divisions is fundamentally disturbed at the early or mid-blastula stage, just when nuclear activity as detected by radiation effects begins. The mitotic coefficient during this period of normal development sharply decreases. This too underlines the important changes taking place in the early or mid-blastula.

In conclusion, the periodicity of nuclear activity raises many problems. Does the character of the activity differ between one period and another, as nuclear morphology alone to some extent suggests? Is there qualitative change in activity during any one period? What occurs in the nucleus between the periods of activity: is it then, for instance (besides undertaking the replication required for mitosis), accumulating the raw material for its next active period and receiving information from the cytoplasm which conditions the nature of the succeeding activity? Is there a periodicity at later stages of development, interruption of activity occurring, for instance, during the critical transitions implied in Waddington's (1957) epigenetic landscape? These are some of the immediate questions for which adequate methods of investigation need to be developed.

SUMMARY

1. The effect of high radiation doses (20–60 kr.) on different stages of the early development of the loach (*Misgurnus fossilis* L.) was studied. Analysis of dose dependence shows that a radio-sensitive component (presumed to be the nuclei) is affected maximally by such doses, whereas a radio-resistant component (presumed to be the cytoplasm) is not affected significantly. The use of such radiation doses is, therefore, a means of investigating the role of nuclei in development.

2. Irradiation with these doses at any time from fertilization to early blastula leads to the arrest of development at one stage—the late blastula—and to death some hours later. Irradiation at any time during the period from late blastula to mid-gastrula similarly leads to arrest of development at one stage—the end of gastrulation. Irradiation during the period from early to late blastula results in the arrest of development at different stages of gastrulation depending on the moment of irradiation. Irradiation after the mid-gastrula results in the arrest of

development at different stages of organogenesis, depending also on the moment of irradiation.

3. The results cannot be explained by changes of nuclear sensitivity, nor by non-specific critical periods in development. They can be explained by changes in the role of nuclei in development, by periods of specific dependence on the function of nuclei.

4. The independence of the stage of developmental arrest on the stage of irradiation, when irradiating from fertilization up to early blastula and from late blastula up to mid-gastrula, shows the relative inactivity of nuclei during these periods of normal development. The strong dependence of the stage of development reached at arrest on the stage of irradiation, when irradiating the early to late blastula and after the mid-gastrula stages, demonstrates the activity of nuclei in development during these periods.

5. The conclusion may be drawn that in the early development of the loach the nuclei function periodically, each period of activity ensuring a subsequent episode of development. Nuclear activity during ovogenesis ensures the development of the egg up to the stage of the late blastula; nuclear activity during the early and mid-blastula stages ensures gastrulation. Development of axial organs and further organogenesis is ensured by nuclear function beginning from the mid-gastrula stage.

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The First Appearance of Specific Antigens during the Induction of the Lens[1]

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INTRODUCTION

THE formation of the lens in the chick embryo is known to depend upon 'inductive' influences from the eye-cup (Alexander, 1937; Van Deth, 1940; Waddington & Cohen, 1936). A period of direct contact between eye-cup and presumptive lens ectoderm from the 9- to the 20-somite stage is essential for the induction (Weiss, 1947; McKeehan, 1951; Langman, 1956). At the beginning of this period (9–12-somite stage), the cytoplasm of the presumptive lens ectoderm cells is vacuolated and the nuclei have a random distribution, as in the ectodermal epithelium of the head region. During subsequent development (13–16-somite stage) the intracellular vacuoles disappear from the presumptive lens ectoderm and the nuclei become gradually displaced toward the base of the cells in contact with the retinal surface (McKeehan, 1951). At the 16–19-somite stage the cells become more and more columnar (so-called palisading phenomenon) and the nuclei elongated perpendicularly to the basement membrane (lens placode formation). According to Weiss (1947) this nuclear orientation suggests molecular orientation (perhaps as a result of the fixation of a protein molecule from the optic vesicle to the basal surface of the presumptive lens ectoderm).

After the lens placode has been formed, its invagination begins (20–22-somite stage, 44–50 hours of incubation) and leads gradually to the formation of a lens vesicle, which is separated from the ectoderm at the 31–34-somite stage (63–72 hours of incubation). Soon thereafter the cells in the posterior wall of the lens vesicle become elongated and form the first lens fibres (34–41-somite stage, 72–96 hours of incubation). At the end of the 5th day a group of tall cylindrical cells found at the equator of the lens turn 130 degrees and form new lens fibres, which join peripherally the central fibres. This marginal zone formation reaches completion at the 8th day.

To determine at what stage of morphological development chemical substances characteristic for the adult lens appear, Burke *et al.* (1944) applied the

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complement-fixation technique and were able to detect the presence of lens antigens in 146-hour embryos, that is, at a time when the first marginal lens fibres have been formed, but not earlier. Ten Cate & Van Doorenmaalen (1950), applying microprecipitin tests, found lens antigens as early as the 30-somite stage, when the lens vesicle is still open to the surface. Langman, Schalekamp, Kuijken, & Veen (1957) searched for lens antigens in chick embryos from 7 to 20 somites by explanting presumptive lens ectoderm with the associated eye-cup into a medium which contained lens antibodies. Degeneration of presumptive lens ectoderm was found in explants cultured in this medium, but not in controls.

In the present work, these experiments were repeated under different experimental conditions using presumptive lens ectoderm not only *with*, but also *without* the associated eye-cup. The latter procedure made it possible to avoid the inductive influence of the eye-cup during the explantation period and to examine whether the first lens antigens appear before or during the contact period. In addition, these experiments gave information on the relationship between chemical and morphological differentiation in presumptive lens ectoderm cells.

MATERIAL AND METHODS

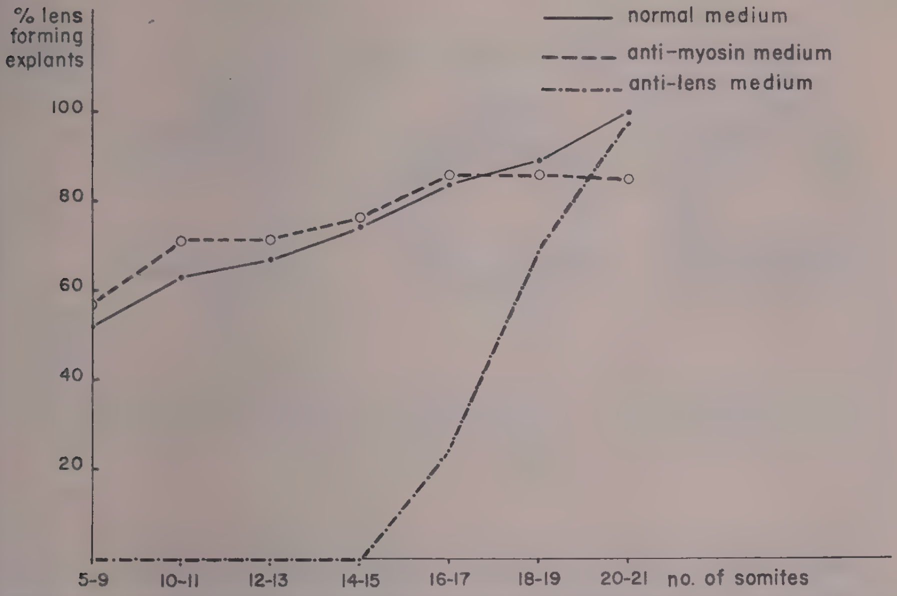
In a first series of experiments, eye-cup material covered with presumptive lens ectoderm obtained from White Leghorn chick embryos of 5–20 somites was explanted in a fluid medium composed of equal parts of $8\frac{1}{2}$-day chick embryo extract, Tyrode solution, and rabbit serum containing antibodies against adult lenses. The antiserum was prepared by injecting chicken lens extract (10 per cent.) into healthy rabbits, using an injection schedule devised by Ten Cate & Van Doorenmaalen (1950). In the control series the anti-lens serum was replaced either by serum of normal healthy rabbits (normal serum) or by serum of rabbits injected with the myosin fraction of frog muscle (DeHaan, 1954).

In the second series of experiments, presumptive lens ectoderm tissue from 7–20-somite chick embryos was dissected free from the eye-cup. The first attempts to do so resulted in damage to the presumptive lens ectoderm due to difficulties in separating the tightly bound ectoderm and eye-cup. Success was achieved after dipping the heads of the embryos in trypsin solution (1:10,000) for 1 minute and rinsing in Tyrode solution. It was then possible to dissect the presumptive lens ectoderm without damaging the cells. The ectoderm cells were explanted on a clot which had the same constituents as the experimental medium mentioned above, but to which an equal amount of chicken plasma was added in order to obtain a coagulum. In the control series the anti-lens serum was replaced by 'normal' serum.

The titre of the lens antiserum used in these experiments was determined by Boyd's method and found to vary from 1:6,400 to 1:16,000. To determine the specificity of the lens antiserum it was tested with extracts from a variety of organs and tissues of adult chickens. No precipitin reaction was found with most organs and tissues used, except with iris, retina, cornea, and vitreous body (Langman *et al.*, 1957).

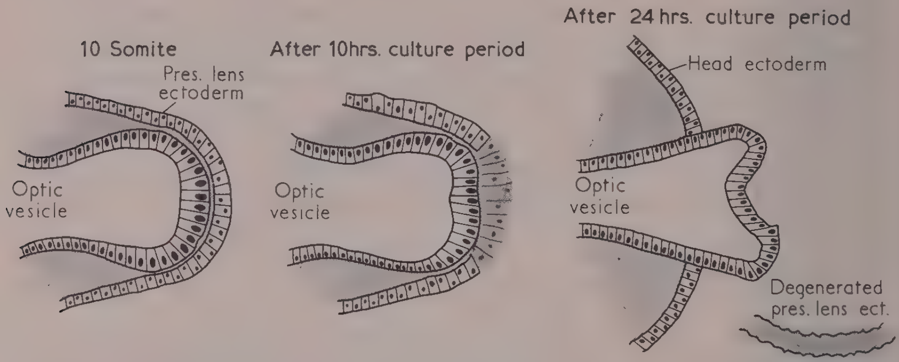
RESULTS

Presumptive lens ectoderm with associated eye-cup cultured in 'normal' and 'anti-myosin' medium for 72 hours gave rise to lens formation in most cases (Text-fig. 1). The percentage of lens-forming explants was found to increase with the age of the embryo from which the tissue is obtained. Failure of lens formation in some of the explants cultured in the control media may be due to mechanical and thermal damage during dissection and heterologous constituents in the culture medium (Langman, 1953 *a*, *b*). Microscopic study of the explants cultured in control media showed a normal, although delayed, morphological development of the lens in comparison with *in vivo* development. Furthermore, the explants cultured in 'anti-myosin' medium showed almost no mesenchyme between the ectodermal derivatives, or only some necrotic cellular material. Lens, optic cup, and surface ectoderm were normal.



TEXT-FIG. 1. Percentage of lenses developing in explants of optic vesicle and presumptive lens ectoderm in various media. The horizontal axis represents the age of embryo at explantation.

The explants cultured in 'anti-lens' medium for 72 hours behaved differently. In studying these tissues during the explantation period under the dissecting microscope, it was observed that 10–20 hours after the beginning of the tissue-culture period the explants showed a white, degenerating area in the presumptive lens ectoderm. This area became larger during the next few hours and often broke off from the explant. Thus in some of the cultures the uncovered optic vesicle protruded through a collar of non-presumptive lens ectoderm, whereas the necrotic presumptive lens area dropped to the bottom of the container (Text-fig. 2). However, it may be seen from Text-fig. 1 that this pattern was not followed by all explants. All those obtained from 5–16-somite embryos were unable to form a lens in anti-lens medium, those from 16 to 17 somites developed a lens in 24 per cent. of the cases, while the explants from embryos of 18 and more somites behaved like the controls and formed a lens.

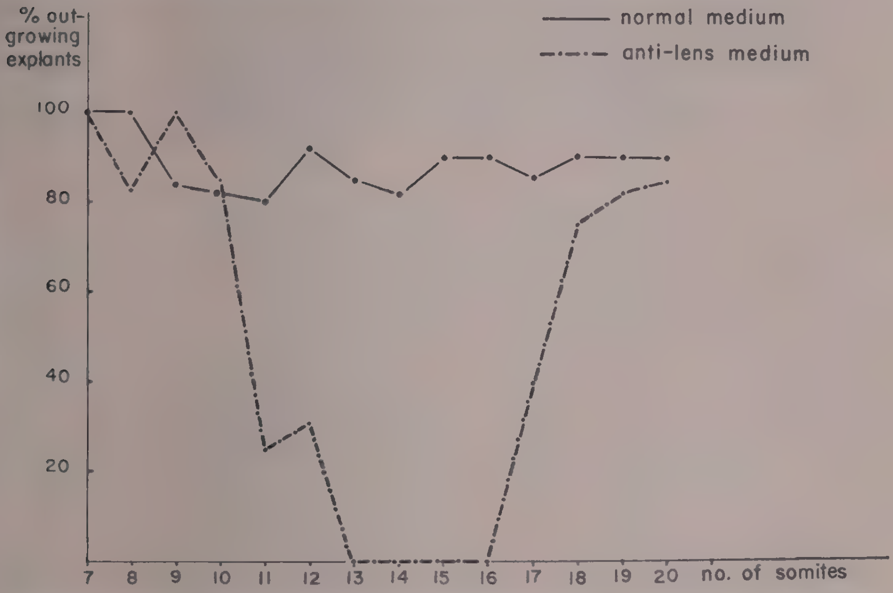

10 Somite
Pres. lens ectoderm
Optic vesicle
After 10hrs. culture period
Optic vesicle
After 24 hrs. culture period
Head ectoderm
Optic vesicle
Degenerated pres. lens ect.

TEXT-FIG. 2. Diagram of changes in explants of optic vesicle and presumptive lens ectoderm from 10-somite embryos cultured in 'anti-lens' medium. On the left, at explantation. Middle, degenerating presumptive lens area after 10 hours. On the right, degenerate lens area lost, optic vesicle protruding through ectoderm.

This experiment, which exactly confirms under our present laboratory conditions a series of data previously obtained (Langman *et al.*, 1957), indicates that 'normal' and 'anti-myosin' media do not interfere with lens formation *in vitro*; 'anti-lens' medium, however, prevents lens development in explants obtained from chick embryos of 5–17 somites. Presumably the lens antibodies present in the culture medium are able to react with constituents of the presumptive lens ectoderm cells in such a manner that the cells are unable to survive.

This experiment did not decisively show whether lens antigens arise during or before the induction period (9–20 somites). It was possible that lens antigens were not yet present in the presumptive lens ectoderm cells of a 7-somite embryo but arose under the influence of the associated eye-cup at some time during the explantation period. The result of a 72-hour explantation period in an 'anti-lens' medium would then be the same, namely, degeneration of the presumptive lens ectoderm. Presumptive lens ectoderm from 7–20-somite embryos was therefore dissected free from the eye-cup and explanted on a clot. By doing so it was possible to exclude the inductive influence of the eye-cup during the 72-hour culture period.

Presumptive lens ectoderm explants without associated eye-cup obtained from embryos of 7–20 somites were studied under the microscope during the explantation period. Twelve to twenty-four hours after the beginning of the culture period explants on 'normal' medium showed migration and outgrowth in the form of a membrane in or on the medium. At the end of the explantation period 80–100 per cent. of the explants showed outgrowth, whatever the age of the donors, that is to say, regardless of whether or not the ectoderm had been exposed to the inductive influence of the eye-cup prior to explantation. However, although the cells looked healthy and migrated, no lenses were formed.



TEXT-FIG. 3. Percentage of explants of presumptive lens ectoderm (optic vesicle removed) showing outgrowth after 72 hours in culture in normal or 'anti-lens' medium. The horizontal axis represents the age of embryo at explantation.

The explants grown on 'anti-lens' medium behaved differently according to the age of the embryos. Presumptive lens ectoderm obtained from 7–10-somite embryos showed outgrowth as explants in normal medium. Cultures obtained from 11–12-somite embryos showed outgrowth only in about 30 per cent. of the cases and those from 13–16-somite embryos in none. In the latter, disintegration and death of the cells were observed in all cases 12–24 hours after the beginning of the culture period. Fragments obtained from embryos beyond the 17-somite stage again showed outgrowth and migration just as the control explants did (Text-fig. 3; Table 1).

From this experiment it can be concluded that, shortly after the 9–10-somite stage, when direct contact between eye-cup and ectoderm is established and induction is thus elicited, the cells lose their ability to survive in 'anti-lens' medium.

A new component somehow appears in the presumptive lens ectoderm cells and reacts with the lens antibodies in the medium in such a way that the cells are unable to survive. Toward the end of the induction period (18 somites), when adhesion between eye-cup and ectoderm is diminishing, the presumptive lens cells are no longer killed by lens antibodies.

TABLE 1

Influence of 'normal' and 'anti-lens' medium on presumptive lens ectoderm

Number of somites	*Number of explants*—$\frac{\text{outgrowing}}{\text{total}}$	
	Normal medium	*Anti-lens medium*
7	$\frac{6}{6}$	$\frac{6}{6}$
8	$\frac{7}{7}$	$\frac{5}{6}$
9	$\frac{5}{6}$	$\frac{7}{7}$
10	$\frac{9}{11}$	$\frac{6}{7}$
11	$\frac{8}{10}$	$\frac{2}{8}$
12	$\frac{12}{13}$	$\frac{4}{12}$
13	$\frac{11}{13}$	$\frac{0}{11}$
14	$\frac{9}{11}$	$\frac{0}{15}$
15	$\frac{10}{11}$	$\frac{0}{20}$
16	$\frac{10}{11}$	$\frac{0}{20}$
17	$\frac{11}{13}$	$\frac{8}{20}$
18	$\frac{9}{10}$	$\frac{9}{12}$
19	$\frac{10}{11}$	$\frac{13}{16}$
20	$\frac{10}{11}$	$\frac{11}{13}$

DISCUSSION

Antibodies produced by injecting specific substances—antigens—into rabbits have the capacity to react selectively with these antigens and with closely related substances. This reaction, which is based upon the formation of an antibody-antigen complex, can become visible as a flocculation, agglutination, precipitate, cell degeneration, &c. By testing extracts of unknown composition with antibodies prepared against a well-defined organ, it is possible to detect whether or not antigens specific for this organ are present in the unknown extracts. Applying this immunological detection method in embryology, it was found that antibodies prepared with antigens of adult organs and tissues may react with antigenically active materials in embryos—a fact indicating that embryonic organs and tissues may possess antigenic substances which are identical or closely related to adult antigens. Some of these embryonic substances persist up to the adult stage, while others are transitory and disappear during development (Ebert, 1953; Tyler, 1955; Nace, 1955).

7–10-somite stage

By culturing presumptive lens ectoderm without the associated eye-cup on a medium containing lens antibodies it was found that explants obtained from 7–10-somite embryos showed the same active outgrowth and migration as the controls (Text-fig. 3). Thus at this stage of development no antigenic substances capable of reacting with lens antibodies were detectable. Examination of presumptive lens ectoderm at this stage using the agar gel technique of Ouchterlony (1948) showed no lens antigens either (unpublished data). Although it is known that the eye-cup has started exerting its inductive influence upon the presumptive lens ectoderm cells at the 9–10-somite stage these cells do not yet contain any chemicals related to lens proteins. Since there is no morphological distinction from the surrounding head ectoderm either, it may be concluded that no detectable chemical or morphological differentiation has yet taken place.

11–17-somite stage

As shown in the introduction, the cells of the presumptive lens ectoderm start morphological differentiation at the 13-somite stage. Explantation of presumptive lens ectoderm *with* or *without* the associated eye-cup from 11–17-somite embryos in a medium containing lens antibodies produced degeneration of the cells (Text-figs. 1 and 3). This result means that substances able to react with lens antibodies arose in the presumptive lens ectoderm at the 11-somite stage, that is, shortly before the cells become distinguishable from the surrounding head ectoderm cells. Hence the first lens antigens seemed to be formed shortly before the first features of morphological differentiation appeared (13 somites).

The lens antisera used in our experiments were prepared with 'adult' lens material. Does this mean that the antigens detected in presumptive lens cells by use of these antisera were the same as in the adult? In testing the presence of lens antigen in chick embryos by means of the precipitin method, Ten Cate & Van Doorenmaalen (1950) concluded that 'adult' lens antigen was present at the 30-somite stage. However, since Landsteiner (1947) found that the specificity of antigenic structures may be represented by relatively small chemical groups on a large molecule rather than by the molecule as a whole, the presumptive lens cells need not necessarily have identical antigens, but merely identical chemical groups.

The fact that morphological lens differentiation was preceded by the appearance of specific chemical units suggests that, under the inductive influence of the eye-cup, some components of the indifferent multi-potent head ectoderm cells acquired the determining groups characteristic of lens antigens. These antigens appearing at the 11-somite stage (first-stage antigens) may give rise to a number of antigenic structures, each preceding the appearance of a new morphological structure as the placode transforms through lens vesicle to adult lens. François *et al.* (1956) showed by immunochemical analysis of extract of adult lens material that at least eight different antigens are present at the adult stage.

A possible mechanism by which antibodies kill presumptive lens ectoderm cells during the 11–17-somite stage may be a cell surface reaction. This antigen-antibody surface reaction may interfere with exchange processes between the cell and its environment, or cause some other change in surface properties (*surface effect*). Another possibility is the passage of antibodies through the cell membrane and the combination with substances within the cell (*intracellular effect*). The antibody-antigen binding may block the function of a cell substance essential for cell growth and survival, or the formed complex may be toxic and thus kill the cell. The entry of the antibody into the cell presumed to occur during the 11–17-somite stage may be an indication of a high permeability of the cell membrane.

18- and more than 18-somite stage

At this stage of development, when the 'first stage' lens antigens have been formed, the differentiation of the lens cells can proceed without any further need for the eye-cup. This was demonstrated by McKeehan (1954), who transplanted placode cells of a 21-somite embryo into the coelomic cavity of another chick embryo. This transplant showed the capacity to form a small lens, indicating independent self-differentiation at this stage of development. Similarly, it appeared from our experiments that at the 18-somite stage the future lens cells are no longer sensitive to lens antibodies (Text-figs. 1 and 3). A possible explanation was that the 'first stage' lens antigens were *transitional antigens*. This was unlikely since, using the same antilens serum but a different technique (Ouchterlony, 1948) we demonstrated the presence of lens antigens at the 18–22-somite stage (unpublished).

An alternative explanation is that the 'surface effect' no longer takes place, perhaps because of a change in the cell membrane. That there are indeed changes in the cell membrane is suggested by the fact that at the 18-somite stage the presumptive lens cells can be separated from the eye-cup without treatment by trypsin solution. This change in the cell membrane may also prevent the access of antibodies into the cells and thus avoid their 'intracellular effect'. If, however, the intracellular reaction is not blocked, it may be that the neutralized antigens are no longer toxic to cell life or, if the original antigens were essential, they can now be replaced by other substances.

From these experiments it may be concluded that lens antibodies applied in tissue culture can act as sensitive indicators of the formation or presence of specific substances (lens antigens) in embryonic cells. The formation of an antigen-antibody complex leads to the death of the presumptive lens ectoderm cells only during the 11–17-somite stage. Before and after this stage the cells are not visibly affected by the presence of lens antibodies, indicating either that the reacting antigens are not present or that the formation of an antigen-antibody complex is prevented. The most likely mode of antibody action is considered to be the 'intracellular effect', the cell membrane, however, having an important function in admitting the antibodies into the cells.

SUMMARY

1. Presumptive lens ectoderm *with* associated eye-cup from 5–17-somite chick embryos was explanted in 'normal' medium, 'anti-myosin' medium, and a medium containing lens antibodies. The control explants in 'normal' and 'anti-myosin' medium showed lens formation in 80–100 per cent. of the cases (Text-fig. 1); the explants in 'anti-lens' medium showed degeneration and necrosis of the future lens area, indicating the presence of substances (lens antigens) capable of reacting with lens antibodies (Text-fig. 1). Explants from embryos with 18 or more somites in 'anti-lens' medium formed lenses as the controls.

2. Presumptive lens ectoderm *without* the associated eye-cup from 7–20-somite embryos was cultured on a 'normal' and 'anti-lens' medium. The controls showed epithelial membranous outgrowth in 80–100 per cent. of the explants (Text-fig. 3; Table 1). Explants on 'anti-lens' medium showed outgrowth similar to controls when obtained from embryos younger than 11 and older than 17 somites, but showed degeneration when obtained from 11- to 17-somite embryos, indicating the formation of the first reacting substances (lens antigens) at the 11-somite stage (Text-fig. 3; Table 1).

3. The 'first-stage' lens antigens are formed at the 11-somite stage, shortly after the adhesion between eye-cup and presumptive lens ectoderm has been established (9 somites) and shortly before the morphological changes characterized by loss of vacuolization, nuclear orientation, and palisade phenomenon appear (13–19 somites).

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The Passage of Urea and Creatinine across the Placenta of the Pig

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WITH ONE PLATE

IT is generally considered that urea diffuses freely across the placental barrier and that creatinine does so too (Needham, 1931), and that the concentration of both compounds is the same in foetal and maternal plasma. Alexander, Nixon, Widdas, & Wohlzogen (1958), however, found that the concentration of creatinine was higher in the serum of the foetal lamb than it was in the maternal serum.

TABLE 1

The concentration of creatinine in the serum of the sow during farrowing, and of her piglets at birth

	Serum creatinine (mg./100 ml.)
Sow 1	2·35
Piglet *a*	4·60
b	7·85
c	3·72
d	3·90
e	5·50
Sow 2	2·43
Piglet *a*	7·42
b	3·38

The authors made no specific comment upon this. McCance & Widdowson (1953) discussed the difficulties which would arise if all the chromogens reacting with alkaline picrate in human cord serum were accepted as creatinine, and in work still to be published these two authors found that the concentration of creatinine 'chromogens' in the serum of the foetal pig at term was always higher than in the maternal serum, although the concentrations of urea were the same.

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Needham (1931) gave no information about this species in his review. McCance & Widdowson further observed that, while the concentration of urea in the serum of the suckled new-born piglet rose in the first few days of life, the concentration of creatinine chromogens fell. The nature and extent of these changes are illustrated in Tables 1 and 2. The figures in Table 2 are the averages for two piglets in each litter. A rise in serum urea is normal at this time of life in many species, including man (McCance & Widdowson, 1947; McCance & Otley, 1951; Boylan, Colbourn, & McCance, 1958; Joppich & Wolf, 1958), but the creatinine chromogens have not been studied to the same extent. Nevertheless, Boylan, Colbourn, & McCance (1958) found relationships at and after birth in the guinea-pig similar to those shown for the pig in Table 2. They did not, however, find concentrations of creatinine in the sera of foetal guinea-pigs to be higher than in those of the mother.

TABLE 2

Changes in the concentration of creatinine and urea in the serum of piglets during the first days of life

Litter	*Newborn*		*1–3 days old*	
	Creatinine (mg./100 ml.)	*Urea* (mg./100 ml.)	*Creatinine* (mg./100 ml.)	*Urea* (mg./100 ml.)
1	2·40	30·0	1·40	94·6
2	4·25	20·3	1·35	62·2
3	3·17	22·6	1·57	92·8
4	2·35	21·4	1·38	145·0
5	2·93	21·6	1·72	65·8

These observations led to the present study in pigs of the chromogens in the foetal and maternal plasma which react with picrate in alkaline solution. The need for such an investigation was suggested also by the finding of Boylan *et al.* (1958) that the concentration of creatinine chromogens in the urine of the foetal guinea-pig near term was sometimes so high that it exceeded the concentration of urea.

METHODS

Most of the animals were reared on the laboratory farm and have been described before (McCance & Widdowson, 1956), but two litters of pure bred 'Large Whites' were investigated through the co-operation of the staff at the Agricultural Research Council's farm at Babraham. Blood was removed from the piglets at birth by heart puncture or by snipping the umbilical cord; the serum was separated within 1–2 hours and stored at —20° C. The sow was bled from the ear during the early stages of labour or while farrowing and her blood treated in the same way. The figures given in this paper refer only to blood removed during farrowing, since there appears to be a small rise in the concentration both of creatinine and urea between the onset of labour and farrowing.

The serum was prepared for chromatography by passing it through an ultrafiltration shell (Membranfiltergesellschaft-Sartorius A.G., Göttingen) using a suction pressure of about 600 mm. Hg., and the salts were removed from the ultrafiltrate by the technique described by Dent (1951). 0·5 ml. of the product were submitted to one-dimensional descending chromatography in *n*-butanol-acetic acid-water (60:15:25) for 22 hours at 19 ± 1° C. The papers (Whatman No. 1) were dried at room temperature and sprayed with a mixture of 10 per cent. NaOH in water (1 part) and saturated picric acid (5 parts).

The concentrations of creatinine in serum were determined by the method described by Hawk, Oser, & Summerson (1954). Other substances chemically determined have been urea (Lee & Widdowson, 1937), fructose (Bacon & Bell, 1948), and pyruvic acid (Friedemann & Haugen, 1943).

RESULTS

Chromatographic

A few minutes after spraying the papers an orange spot with an Rf value of 0·51 began to develop. This was often visibly more intense in the serum of the piglet than in that of the sow (Plate, fig. A 2, 3). It was identified as creatinine and did not fade for many days in the absence of light. An hour or so after spraying a browner spot with an Rf value of 0·26 slowly began to develop in piglet sera. This continued to become more intense for at least a week and was identified as fructose by its Rf value and its specific colour reactions on other papers. By this time a weaker spot with a similar colour and an Rf value of 0·21 had appeared in the maternal and foetal sera and was found to be glucose. Photographs of these spots 3 days and one week after spraying are shown in figs. A 2, 3 and B 2, 3 of the Plate. The colours produced by 15 μg. of creatinine, 180 μg. of fructose, and 120 μg. of glucose are also shown in figs. A 4 and B 4, and figs. A 5 and B 5 give the reactions of a human cord serum to which 120 μg. of fructose had been added. Fructose was not detected in the serum of the sow. No chromogens other than creatinine and fructose were found in piglet serum treated in the way described. It is not easy to separate glycocyamidine from creatinine in secondary butanol solvents (Maw, 1948), but this substance is not generally considered to be a biological precursor of creatinine and it has an Rf value sufficiently different from that of creatinine to make a band of colour rather than a spot when present in the same solution. It also reacts more slowly. It is, therefore, not considered likely to have been present in appreciable amounts.

Pyruvic acid was not detected in either foetal or maternal sera although it could be determined chemically in both (see below). This was presumably because it was oxidized on the paper, or during the deproteinization and desalting of the sera in preparation for chromatography. Plate, figs. A 1 and B 1 show the spot produced by 25 μg. of Na pyruvate in water. Koštíř & Rábek (1950) obtained a spot on their chromatograms by the addition of 6 μg., but Maw (1948) found it necessary to add 100 μg.

Chemical

Table 3 shows the concentrations of creatinine chromogens, fructose, and pyruvic acid in the serum of four piglets, two from each of two litters, within a few moments of birth and again when they were 24 hours old. The serum of both sows contained approximately 2·4 mg. creatinine chromogens, 2 mg. fructose, and 2 mg. pyruvic acid per 100 ml. The piglet's serum, therefore, had a considerably higher concentration of creatinine chromogens than that of the sow, but within 24 hours the figures were at the adult level. It is probable that some of the figures for serum creatinine in the new-born pig shown in Table 2 were already lower than they had been at the moment of birth, for some of the samples were collected by heart-puncture an hour or more after the animals were born. The

TABLE 3

Creatinine, fructose, and pyruvic acid in the serum of 4 piglets at birth and again 24 hours later

Piglet		*Creatinine* (mg./100 ml.)	*Fructose* (mg./100 ml.)	*Pyruvic acid* (mg./100 ml.)
At birth	1	4·02	49·3	1·7
	2	3·35	34·2	1·7
	3	7·42	40·2	1·6
	4	3·38	49·8	2·7
24 hours old	1	1·63	3·3	2·1
	2	2·13	2·5	2·7
	3	2·20	2·7	4·0
	4	2·54	2·1	4·2

TABLE 4

The effect of pyruvic acid and fructose on the colour produced by alkaline picrate

	Apparent creatinine (mg. per 100 ml.)	*Difference due to pyruvic acid or fructose*
Serum from day-old piglet alone	1·80	—
Serum + pyruvic acid 2 mg. per 100 ml.	2·03	0·23
Serum + fructose 50 mg. per 100 ml.	1·94	0·14
Serum + pyruvic acid 2 mg. + fructose 50 mg. per 100 ml.	2·14	0·34
Creatinine solution alone	3·00	—
Creatinine + pyruvic acid 2 mg. per 100 ml.	3·23	0·23
Creatinine + fructose 50 mg. per 100 ml.	3·12	0·12
Creatinine + pyruvic acid 2 mg. + fructose 50 mg. per 100 ml.	3·33	0·33

amounts of pyruvic acid found in the serum of the piglet and sow were about the same, and the concentration of pyruvate tended to rise during the first 24 hours of life. The results for fructose confirm Goodwin's (1957) observation that the concentration in serum is high at birth but falls rapidly afterwards.

Table 4 shows the effect of adding physiological concentrations of pyruvic acid and fructose on the colour produced by creatinine in alkaline sodium picrate. The effect of pyruvic acid was much smaller than that observed by Koštíř & Sonka (1952) and it is clear that neither substance could have accounted for the difference between the 'creatinine' values of piglet and sow sera, or for the fall in these values in piglet sera after birth. Glycocyamine gave no colour at all with alkaline sodium picrate.

DISCUSSION

The evidence presented above leads to the conclusion that there is normally a greater percentage of creatinine in the serum of the foetal pig at term than there is in the maternal serum. The same is probably true of the sheep (Alexander, Nixon, Widdas, & Wohlzogen, 1958). This animal is also an ungulate, but it has a very different placenta, and it looks as though the small molecule of creatinine must be added to others, such as fructose and some of the amino-acids, which are not equally distributed on either side of the placenta in all mammals. There is nothing new in the finding that some cells in the body may be much less 'permeable' to creatinine than to urea, for such cells must exist in the kidney, but the physico-chemical basis is unknown. One further point of possible significance has emerged from this investigation, and that is that, within one and the same litter, one piglet may have a creatinine value in its serum not much higher than that of the sow, whereas another may have a value over three times as great (Table 1). There may be other great differences between the foetal fluids and membranes of litter-mate piglets (Wislocki, 1935), but no explanation has been forthcoming.

SUMMARY

1. The concentration of 'creatinine chromogens' in the serum of the foetal pig at term may be over three times that in the maternal serum. The concentrations of urea were always about the same.

2. The values for these chromogens in piglet sera fell to the adult level in the day following birth.

3. Fructose was the only chromogen other than creatinine found in piglet serum by chromatography.

4. Pyruvic acid was not the cause of the difference.

5. It is concluded, therefore, that the chromogens were mostly true creatinine, and that the pig's placenta is less 'permeable' to it than to urea.

ACKNOWLEDGEMENTS

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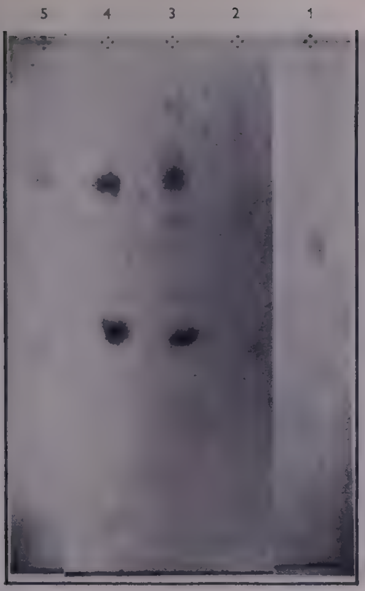
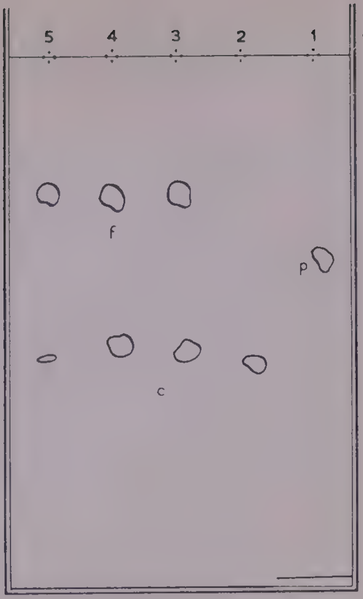
EXPLANATION OF PLATE

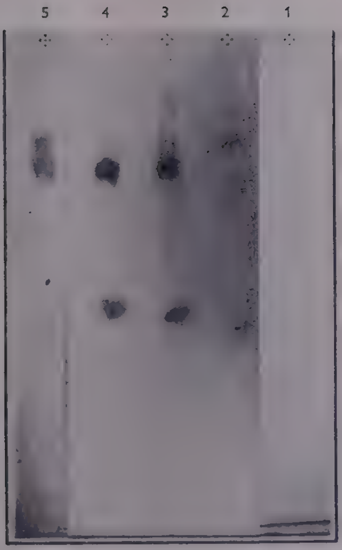
Key: c = creatinine; p = pyruvic acid; f = fructose; g = glucose

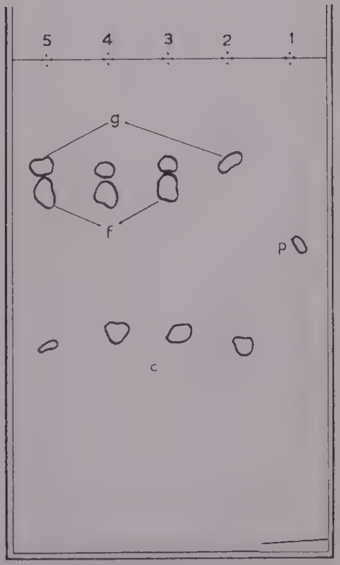
Fig. A. Descending paper chromatogram 3 days after spraying with alkaline picrate. A′ is a drawing providing a key to the identification of spots.

1. Sodium pyruvate (25 μg.). Distinct spot.
2. Sow's serum (0·5 ml. desalted ultrafiltrate). Weak creatinine spot.
3. Piglet serum (0·5 ml. desalted ultrafiltrate). Distinct creatinine and fructose spots.
4. Creatinine (15 μg.). Distinct spot. Fructose (180 μg.). Distinct spot. Glucose (120 μg.). No spot visible.
5. Human cord serum (0·5 ml. desalted ultrafiltrate) with added fructose (120 μg.). Weak creatinine spot, distinct fructose spot, and very weak glucose spot.


A


A′


B


B′

E. ZWEYMÜLLER, E. M. WIDDOWSON, *and* R. A. McCANCE

FIG. B. The same paper chromatogram as shown in fig. A, 7 days after spraying with alkaline picrate. B′ is a drawing providing a key to identification of spots.

1. Pyruvate spot barely visible.
2. Distinct glucose spot.
3. Distinct fructose and weak glucose spots.
4. Distinct fructose and weak glucose spots.
5. Distinct fructose and glucose spots.

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Differentiation of Cells of the *Rana pipiens* Gastrula in Unconditioned Medium[1]

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WITH THREE PLATES

INTRODUCTION

INSTANCES of differentiation in absence of an inductor have multiplied to such extent that the classical picture of embryonic induction is in process of serious re-examination (Grobstein & Parker, 1958; Nieukoop, 1955; Trinkaus & Groves, 1955). Questioned on the basis of the occurrence of neural differentiation without organizer in explants of presumptive epidermis (Barth, 1941; Holtfreter, 1947, 1955; Itô, 1952), the necessity for a specific inductor has proved difficult to demonstrate in other inductive systems. Grobstein & Parker (1958) have shown that tubule formation in metanephrogenic mesenchyme may occur in abnormal sites *in vivo* without contact with its normal ureteric inductor and even in the anterior chamber of the eye without contact with any other tissue. Niu (1958*b*) has recently reported a certain percentage of neural differentiation in explants of presumptive epidermis cultured as controls in his salt medium without addition of inductor substances.

Such observations not only emphasize the difficulty of finding an adequate neutral environment for the study of inductors but also suggest the necessity for revision of such concepts as induction and self-differentiation.

The present experiments were undertaken with the objective of working out a simple system for culturing presumptive epidermis in a situation that would permit easy alteration of the culture medium and facilitate observation of the effects on differentiation of specific additions to the medium. The work of Niu (1956) had demonstrated in elegant fashion the advantages of using very small explants which could attach to glass and spread out in a single cell layer eliminating the need for sectioning. The success of Niu's medium (Niu–Twitty solution) in cultures of *Ambystoma* and *Triturus* explants offered new hope that a similarly modified Holtfreter's solution might support *in vitro* differentiation of early embryonic cells from *Rana*. *R. pipiens* was chosen as the source of presumptive

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epidermis explants because of the relative resistance of this species to neuralization by factors other than the living presumptive chordamesoderm, its normal inductor (Smith & Schechtman, 1954).

The results to be reported in the present paper lend additional support to the idea that presumptive epidermis cells possess a spectrum of differentiation potentialities that may be brought to expression without contact with an organizer or even exposure to a medium conditioned by such an organizer.

EXPERIMENTAL PROCEDURE

Operations

Eggs of *R. pipiens* obtained by pituitary injection were used at early gastrula stages (10 plus to 11 minus of Shumway, 1940). Jelly was removed by means of watchmaker's forceps, and the eggs were passed through three changes of sterile 10 per cent. Ringer's solution. All solutions and instruments used thereafter were sterile. The explants were dissected out in solution *X* (see section on solutions) on 2 per cent. washed Bacto-agar. A median strip extending ventrally from animal pole to a line approximately 60° from the animal pole provided presumptive epidermis explants (regions 3 and 4), while the area just above the dorsal lip of the blastopore (region 1) was used as presumptive chordamesoderm (numbers of regions refer to diagrams in Sze, 1953). In the absence of a precise map of the gastrula of *R. pipiens* we took the precaution of observing the development of the donors of the presumptive epidermis. The opening into the blastocoel healed rapidly at room temperatures and normal appearing late neurulae developed within 20 hours. The small wound marking the site of explantation was located ventrally about midway along the antero-posterior axis of the embryo.

Four explants at a time were transferred by means of a Spemann pipette to the versene dissociating medium in a stender dish with agar-coated bottom. The explants, with pigment coat side down, remained in the dissociating medium for an average time of 5 minutes, after which they were returned to solution *X* on agar. The pigment coat layer then could be peeled off easily with glass needle and hair loop, and the underlying layer of presumptive epidermis cells, loosened by the versene treatment, could be teased apart gently into small aggregates of about 125 cells each. Each explant yields approximately 20 aggregates, with an average diameter of 200 μ. These aggregates became spherical and healed within about 30–60 minutes in solution *X*. They then were plated out on to sterile, round coverslips in small stender dishes containing solution *X*. Such cultures were raised at room temperatures ranging from 24° to 27° C. Daily observations could be made with minimal danger of contamination by covering the compound microscope with a shield of transparent plastic sheeting.

When permanent records of a culture were desired the culture medium was drawn off, and the preparation was washed free of globulin with solution and fixed with aqueous Bouin's solution. Staining with 50 per cent. Harris's haematoxylin and dehydration were carried out with the culture still on a coverslip in the stender dish. Final dehydration, brief counterstaining with alcoholic eosin, and clearing in xylol were done by lifting out the coverslip with fine forceps and passing it through 20 ml. stender dishes containing the alcohols or xylol. The coverslip bearing the culture was inverted over a slide bearing a small drop of mounting medium.

Solutions

1. *Medium for operation and culture*

Preliminary experiments to devise a culture medium for cells of the early *R. pipiens* embryo were based upon modifications of both Holtfreter's solution and Niu–Twitty solution, neither of which proved satisfactory under the conditions of our experiments. The composition of the saline medium used in the present experiments is given in Table 1.

TABLE 1

Composition of solution X

A		*B*		*C*	
NaCl (Merck Biol.)	5·150 g.	$NaHCO_3$	0·200 g.	Na_2HPO_4	0·0300 g.
KCl	0·075 g.				
$MgSO_4.7\ H_2O$	0·204 g.	H_2O to 250 ml.		KH_2PO_4	0·0375 g.
$Ca\ (NO_3)_2.H_2O$	0·062 g.				
$CaCl_2.2\ H_2O$	0·060 g.			H_2O to 250 ml.	
H_2O to 500 ml.					

Double distilled water prepared in a glass still during the second distillation was used throughout. The stock solutions were kept in the refrigerator. Aliquots consisting of 50 ml. of *A* plus 10 ml. of glass distilled water to correct for evaporation during autoclaving; 25 ml. of *B*; and 25 ml. of *C* were autoclaved for 20 minutes at 15 lb. pressure. Before mixing these three aliquots together, 100 mg. of serum globulin (Bios) were added to the flask containing 25 ml. of sterile *C* or *B*, brought to a boil several times over a flame to sterilize, and cooled before addition to the other components.

The addition of the 0·1 per cent. globulin proved essential. Without globulin the aggregates attached to the coverslip but showed signs of injury (fuzzy cell outlines) within 24 hours and failed to differentiate. Other macromolecular constituents tested in preliminary experiments (albumin, fibrinogen, chick embryo extract, tadpole extracts) gave less or no protection.

The inclusion of globulin in the culture solution has made it possible to obtain cell division and differentiation in volumes of solution ranging from 2 to 16 ml. Preliminary experiments showed that small explants of presumptive epidermis cultured in hanging drops of solution *X* without globulin would differentiate and form nerve fibres. When larger volumes were used (2 or more ml.), the explants failed to differentiate unless globulin was added. With added globulin presumptive epidermis will form nerve fibres in our largest culture dishes containing 16 ml. of solution.

The pH of solution X varies from 8·1 to 8·8 with the length of time elapsed after autoclaving during which carbon dioxide is driven off and only gradually replaced by atmospheric carbon dioxide. The final pH of solution *X* in the culture dishes was 8·1.

2. *Dissociating medium*

During the early phases of this investigation a glycine-KOH buffer at pH 9·8 was compared with versene as a dissociating medium. The alkaline buffer (suggested by the work of Townes & Holtfreter, 1955) gave the same results as did versene for dissociation and later behaviour of the aggregates during culture in solution *X*. The necessity of adjusting the pH of the glycine buffer introduced danger of bacterial contamination so that versene finally was settled upon as the standard dissociating medium. It was further established that small aggregates of cells obtained by mechanical teasing with a hair loop differentiated upon culture in a manner entirely comparable with that exhibited by aggregates obtained by alkaline or versene dissociation. The chemical dissociation therefore facilitates dissection without affecting the later differentiation capacities of the cells. Composition of the versene medium is set forth in Table 2.

TABLE 2

Composition of dissociating medium

A		*B*		*C*	
NaCl (Merck Biol.)	6,800 mg.	Na_2HPO_4 anhy.	110 mg.	$NaHCO_3$	200 mg.
KCl	100 mg.	KH_2PO_4	20 mg.		
Versene*	744 mg.				
H_2O to 500 ml.		H_2O to 250 ml.		H_2O to 250 ml.	

* Disodium ethylenediamine tetraacetate (Fisher).

The medium for dissociation thus consists of versene in double 'A' Niu–Twitty solution from which calcium and magnesium have been omitted. Its pH is 7·6. Glass distilled water was used. Aliquots consisting of 50 ml. of *A*, 25 ml. of *B*, and 25 ml. of *C* were autoclaved separately and combined under sterile conditions before use.

OBSERVATIONS AND RESULTS

I. *Differentiation of standard cell types from the gastrula of* R. pipiens

In order to identify cell types under experimental conditions it is obviously necessary to know what given cell types look like under the standard conditions used for culture.

Identification of cell types

Preparations of explants from the dorsal and lateral lips of the blastopore of early gastrulae as well as explants from the chordamesoderm and neural plate of older embryos were cultured in order to provide a basis for the identification of cell types.

Explants of the lateral lip of early stage 11 gastrulae attach to the coverslips and differentiate into nerve-cells with nerve fibres, muscle which shows irregular twitching, pigment cells, and 'giant' cells in 3 days at room temperatures. The cultures show progressive differentiation and maintain themselves for from 10 to 20 days.

Nerve-cell differentiation is shown in Plate 1, fig. 1 in a 4-day culture. The individual fibres originate as outgrowths of neuroblasts as described by Harrison (1910). Many nerve fibres may migrate out together, forming a nerve-trunk from which groups of fibres branch. The fibres often terminate in peripheral cells but sometimes migrate in a straight line over the coverslip without making attachment.

Muscle-cell differentiation is illustrated in Plate 1, fig. 2 in a 5-day preparation. Although this particular cell does show cross-striations, often functional muscle-cells show no cross-striation. The form of muscle-cells varies a great deal, but the cell usually can be recognized by its large size, elongate shape, and the presence of internal fibres after fixation and staining.

In 60 per cent. of the cultures single muscle-cells or groups of muscle-cells were observed to twitch at intervals. A period of several contractions was followed by a period of quiescence for several minutes after which twitching was resumed. Muscle-twitching continued for 6 days at room temperatures, after which time the muscle remained quiescent. The muscle then responded to electrical stimulation for two additional days.

Although 100 per cent. of the cultures contained muscle-cells, only 60 per cent. exhibited muscle-contraction. In the latter group nerve-fibres from an adjacent mass of nerve-cells terminated in the muscle-cells. The non-functional muscle-cell did not receive nerve fibres. These muscle-cells did, however, contract when stimulated electrically.

The above observations suggest that the nerve-cell bodies discharge at irregular intervals and send impulses along the fibre to muscle which responds by twitching. Further evidence of nerve action was obtained when simultaneous muscle-twitching was observed in two or more masses of muscle-cells with a common nerve supply. When nerve fibres migrate and make contact with muscle-cells some of the fibres may continue to migrate and innervate a second group of muscle-cells. When this occurs the two groups of muscle-cells twitch at the same time and remain quiescent for the same period. As many as three separate masses of muscle-cells connected only by nerve fibres have been observed to undergo simultaneous muscle-twitching. When the nerve fibres are scraped from the coverslip with watchmaker's forceps the denervated muscle-mass stops twitching, while the innervated mass continues to contract. A single nerve fibre is sufficient to conduct impulses to a single muscle-cell. Plate 1, fig. 3 shows a single muscle-cell with a single nerve fibre. This cell was the terminal muscle-cell of a chain of three groups of cells showing simultaneous twitching.

Some of the cells from lateral lip explants behave in a different fashion from presumptive nerve- and muscle-cells. While the latter form masses, other cells spread out as individual cells and assume shapes varying about the stellate form. On the third day very distinctive slate-grey pigment granules form a pigmented ring near the nucleus. After 9 days of culture these cells assume the shape of pigment cells. The description of differentiation of pigment cells will be given in more detail in the section dealing with the effects of lithium chloride.

Another type of cell has been termed a 'giant' cell. It is characterized by its large size and large nucleus as compared with a neuroblast. The term giant cell is inappropriate since these cells are simply so flattened against the coverslip that the area of the cell and nucleus is greater than in a spherical cell. We have not been able to ascertain the fate of these cells with any certainty. However, on the 6th–7th days a new formation of cells appears. Elongate cells line up in parallel fashion to form a sheet one cell in thickness. The sheet increases in surface area with time, and it may be that the giant cells contribute to it. Mitotic figures are present in the stained sheet of cells. Plate 1, fig. 4 illustrates a portion of a sheet of elongate cells after staining. The cell type is termed *X* in this paper, since we have not obtained any recognizable cell type from such cells.

Although dorsal and lateral lip explants from the early gastrula do contain presumptive notochord cells, we have found only occasional differentiation of notochord. Within the mass of cells which forms nerve fibres small clumps of larger cells with very clear cytoplasm may be diagnosed as notochord cells.

Our study of the stained preparations is by no means exhaustive and possibly we have overlooked other cell types present. Our main objective was to identify a few common cell types for comparison with our experiments with the presumptive epidermis.

Cellular differentiation from presumptive epidermis

Explants of the presumptive epidermis, prepared as described in the section on methods, attach to coverslips and cell migration is under way by the second day at room temperatures. Cells leave the main central mass and spread out in all directions as individual cells and also as a sheet. Since the cells are thin, the surface areas of the nucleus and cytoplasm are large and the cells are termed 'giant' cells. Patches of the sheet are composed of ciliated cells and in some instances the entire mass may fail to attach and instead develop cilia at the surface. These swim about for several days. From attached masses nerve fibres develop on the fourth day. They migrate over the surface of the sheet of giant cells and on to the glass surface where they may continue to migrate or make attachment to individual cells in the periphery. The central mass of cells containing neuroblasts continues to send out nerve fibres, and by the 6th day (Plate 2, fig. 5) many individual nerve fibres are present, as well as bundles of fibres. The fibres branch and also form networks. Many fibres terminate in peripheral cells.

On the seventh day elongate cells with elongate nuclei appear and the cells line up in parallel fashion. The sheet of unoriented cells with circular or elipsoidal nuclei forms wedge-shaped patterns of oriented cells at the periphery (Plate 2, fig. 6). The parallel alignment of cells then spreads throughout the entire sheet. The appearance of the cell which we call *X* is shown in Plate 1, fig. 4 after 3 weeks at room temperature.

While the sheet of cells is undergoing the transformation described above, the nerve fibres become detached from the sheet and later from the glass surface and finally the central mass containing cell-bodies loosens and floats off. As a result, only the oriented sheet of cells and a few detached cells are left on the coverslip after about 10 days of culture. No significant changes can be seen between 10 days and 3 weeks of culture.

Study of the living cultures and of the stained preparations have given no evidence of muscle, pigment, or notochord cells from presumptive epidermis.

The fate of small explants from the outer pigmented layer of epidermis was followed for several days in culture. The cells become ciliated and long streamers of mucus are exuded from the explant. Generally the explant does not attach to the coverslip. While the inner layer requires globulin for survival, the outer layer lives well in solution *X* without globulin. Similarly, the wounds in the early stage 11 donors of the presumptive epidermis heal well and gastrulation and neurulation occur in solution *X* without globulin. Globulin therefore appears to be necessary only for survival of cells from the inner layer of the gastrula.

II. *The effect of lithium upon differentiation of presumptive epidermis*

Lithium chloride was added to solution *B* and boiled briefly to sterilize before mixing with *A* and *C* containing 100 mg. of globulin. The final concentration of lithium chloride was 0·1 M in the standard solution *X* with globulin and the pH of the mixture was 8·5. Following operation in the standard solution *X*, aggregates were immersed in the lithium solution for intervals varying from 1 to 5 hours after which they were plated out on to coverslips in the standard solution *X* for culture. Occasional experiments were made using long duration (18–19 hours) of lithium treatment at low concentration (0·005 M LiCl). The results did not differ from those obtained with the short lithium treatment.

A difference in behaviour of aggregates treated with lithium from those not so treated becomes apparent as early as the second day. The aggregates spread out completely on the coverslips as flat sheets of cells; only rarely does a small clump remain. On the third day, by which time control whole embryos kept at the same temperature have reached hatching (S 20), the aggregates have become massive sheets of about 100 flat, semi-detached giant cells each. By the fourth day an accumulation of pigment in each cell, that is sometimes apparent even on the third day, is a typical feature of these lithium-treated cells. Near the nucleus an accumulation of pigment granules forms a slate-coloured ring in the cytoplasm (Plate 2, fig. 7). This type of cell was never observed in standard presumptive epidermis cultures, although pigment-ring cells were common in cultures of presumptive chordamesoderm in solution *X*.

From the fifth day on there occurs a progressive transformation of the pigment-ring cells into a variety of cell shapes. These changes occur first at the edges of the large sheets of ring cells and by 14–16 days only a few ring cells remain, the rest having assumed some of the following shapes. Dendritic and spindle-shaped cells are found (Plate 2, fig. 8 and Plate 3, fig. 9). In other cells the pigment rings disperse to give uniform pigmentation in large cells with amoeboid processes (Plate 3, fig. 10). In some instances these were seen to become vacuolated and to transform into highly dendritic melanophores (Plate 3, fig. 11).

The effect of lithium is a highly consistent one. When, as described above, the aggregates attach and spread out into a single layer of large cells they undergo 90–100 per cent. transformation into the pigment-ring cells. When an occasional small clump of cells remains at the centre of such a sheet of cells, nerve fibres extending out from the clump are a not infrequent character of later differentiation. The fate of the pigment-ring cells during the second week of culture is varied, as described above, but none of the cell types thus evoked are characteristic of presumptive epidermis cultured in solution *X*.

A suggestive although preliminary result was obtained when the presumptive epidermis explants were treated from 3 to 5 hours with 0·1 M LiCl in solution *X* in which water had been substituted for bicarbonate and carbon dioxide had been bubbled through to a pH of 5·35 before the beginning of the treatment. In addition to the massive transformation into ring cells and their differentiation into the usual spindle-shaped, mesenchyme-like, or dendritic cells typical of lithium cultures, two new types of cells appeared in some of the cultures. Notochord, diagnosed on the basis of its tubular structure and vacuolated cells, was found in some cultures (Plate 3, fig. 12), and in others a cell type characterized by possession of emerald green pigment vacuoles appeared during the second week of culture.

DISCUSSION

The results of the foregoing experiments contribute to the recent revival of the issue of differentiation potentialities of normal presumptive epidermis cells. Cultured in a medium of pure salts, the small explants from the inner layer of presumptive epidermis showed injury and cytolysis and failed to differentiate. Upon the addition of serum globulin what appears to be a protective action of the protein was found, and nerve-cells with nerve fibres differentiated from presumptive epidermis.

Grobstein & Parker (1958) have outlined most succinctly the puzzling problem of the non-specific effects upon self-differentiation of *in vivo* sites. The present results could well be interpreted as a protective or 'sparing' action by the globulin, permitting expression of a wider range of inherent differentiation potentialities. Bautzmann (1929) and Holtfreter (1929) demonstrated neuralization of ectoderm in explants in the cavity of the eye and in the coelomic fluid respectively.

The necessity for 'conditioning' a medium containing serum for culture of cells is universally recognized (e.g. Earle, Bryant, & Schilling, 1954). Most workers provide for such conditioning by using a low ratio of medium to cell number. It is significant that the present experiments demonstrate that upon addition of globulin, the ratio of volume of medium to cells no longer is a critical factor.

Qualitative differences between conditioned media were indicated by Niu's (1956) work. While Niu obtained neural differentiation from presumptive epidermis cultured in medium conditioned for 7–10 days by inductor tissues, he obtained an estimated 73 per cent. myoblast differentiation in medium conditioned for 12 days. The present results show that conditioned medium is not necessary for *neural* differentiation, but we have as yet observed no differentiation of myoblasts from presumptive epidermis, despite the fact that our medium supports the differentiation of functional striated muscle from lateral lips of the blastopore. We feel that the question is still open as to whether different substances or different concentrations of the same substance are required for myoblast differentiation. A recent assay (Fraser, 1957) of the stimulatory effect of various fractions of egg albumin on somite differentiation in the early explanted chick embryo suggests rather complex though still fairly non-specific requirements. For example, alanine and a sulphur-containing protein are among the requirements for somite differentiation.

Another indication that globulin is not entirely adequate to support differentiation under the present conditions of culture comes from the low incidence of notochord in our cultures. This apparent deficiency, however, might be due to the difficulty of diagnosing notochord cells before they become oriented into their characteristic tubular arrangement (Mookerjee, Deuchar, & Waddington, 1953).

The mass transformation of cell type obtained with lithium treatment has two important aspects. First, the lithium effect is a consistent one affecting at least 90 per cent. of the cells under optimal conditions. Secondly, the new cell types produced by lithium differentiate long after the lithium is removed, showing that a permanent transformation has occurred. A disappointing feature of certain other transformed cells has been their reversion to original cell type upon removal of the transforming agent.

The appearance of cells after brief lithium treatment bears a striking resemblance to that pictured and described by Wilde (1955*b*) for ventral ectoderm and neural crest cultures of *Ambystoma* to which phenyl alanine or tyrosine has been added. Wilde noted the appearance of pigment granules first near the nucleus of phenyl alanine-induced melanophores (cf. our pigment-ring cells), confirming Niu's (1954) earlier study.

The mutual repulsion, i.e. spreading and migration, of the lithium-treated presumptive epidermis cells resembles that demonstrated by Twitty (1953) and Twitty & Niu (1954) for pro-pigment cells *in vitro*. The need for oxygen in melanin synthesis (Twitty & Niu, 1954) would be well fulfilled in our experiments where cells are spread out in a thin layer and exposed to a relatively large volume of culture medium. The vacuolization of giant cells during transformation into melanophores pictured by Niu (1954) was observed in our lithium cultures (Plate 3, fig. 11). In our experiments a release of melanogenic chemicals from disintegrating melanophores (Niu, 1954) cannot be involved in the transformation.

As to the biochemical basis for the lithium effect on differentiation of presumptive epidermis, a search of the literature on lithium is of little assistance. Long used by embryologists as an experimental tool (see references to literature in Dent & Sheppard, 1957), the lithium ion has received little attention from biochemists. Some information has come from the work of Ranzi and his colleagues on the effect of lithium on protein structure (Ranzi *et al.*, 1957, cite references to their earlier work). Further information presented by Gustafson (1950) implicates lithium as an inhibitor of respiratory enzymes and protein synthesis. That lithium in the amphibian egg is bound by material other than yolk was demonstrated by autoradiography by Dent & Sheppard (1957), an observation in agreement with the earlier work of Ficq (1954) who found a concentration of lithium associated with melanin granules. The observation in the present experiments that an exposure of as short duration as one hour sufficed to alter the direction of differentiation might indicate that such binding of the ion by some cell structure is a comparatively rapid event.

Although the fact that presumptive epidermis can be neuralized by abnormal treatment of a continuing character was demonstrated by Waddington, Needham, & Brachet (1936), 'shock' treatments of presumptive epidermis that result in neuralization first were described by Holtfreter (1947). Yamada's (1950) extensive studies using ammonia shock treatments furthered the concept that presumptive epidermis could be 'dorsalized' by such non-specific means. Pasteels's (1953) activation of presumptive epidermis by brief centrifugation at low speeds may belong in the same category, since explants of presumptive epidermis made immediately after centrifugation developed neural structures. Flickinger (1957), using carbon dioxide treatment at pH 3·7 for 10–60 minutes, also reported neuralization of presumptive epidermis. The present experiments amplify the findings of these investigators in that the presumptive epidermis is shown capable of neuralization without 'shock' treatment when protected by globulin in the culture medium.

In some interesting recent experiments by Karasaki (1957) it was demonstrated that disaggregation alone by such agents as Ca-free Holtfreter's is not a sufficient condition for dorsalization, a conclusion supported by our own control experiments using mechanical dissociation to obtain small explants. Karasaki suggests that the neuralization brought about by 'shock' treatments (acids and alkalis) may be mediated by changes in the physical structure of the endoplasm manifested by active protoplasmic movements. Lithium in Karasaki's experiments had a suppressive influence upon dorsalization, which he associates with the increase in viscosity of proteins effected by lithium.

In the present experiments also lithium had a suppressive influence upon the differentiation of nerve-cells from presumptive epidermis. Suppression of this capacity for neural differentiation having been brought about by lithium, the presumptive epidermis cells then exhibit a further differentiation potentiality; they can form melanophores and mesenchyme.

SUMMARY

1. A method has been devised for the culture of gastrular cells of *R. pipiens*.

2. Histological and functional differentiation of nerve, muscle, and pigment cells is described as well as the differentiation of ciliated, mucus-secreting, and notochord cells.

3. The inner layer of the presumptive epidermis differentiates into nerve-cells, ciliated cells, and 'giant' cells, in addition to an unidentified cell type *X*.

4. Brief lithium treatment of presumptive epidermis results in a permanent transformation into pigment cells and mesenchyme.

ACKNOWLEDGEMENT

We owe the suggestion of the versene mixture employed for dissociation to the late Dr. Richard Stearns.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. A 4-day culture of the lateral lip of the blastopore of Stage 11 showing 'giant' cells and nerve fibres. High power.

FIG. 2. A 5-day culture of the lateral lip of the blastopore of Stage 11 showing a striated muscle-cell. This cell exhibited muscle-twitching at intervals. High power.

FIG. 3. Muscle-cell from a 6-day culture of dorsal lip of blastopore of Stage 11. The cell possessed four extensions containing fibrils and contracted simultaneously with two nearby groups of muscle-cells. The only nervous connexion to the single muscle-cell is shown in the lower left-hand corner. High power.

FIG. 4. A 3-week preparation of presumptive epidermis of Stage 11 showing the elongate cells with elongate nuclei. Similar preparations are obtained from dorsal lip explants. High power.

PLATE 2

FIG. 5. A 6-day culture of presumptive epidermis of Stage 11 with maximal proliferation of nerve fibres. High power.

FIG. 6. Earliest stage in the formation of elongate cells with elongate nuclei. Presumptive epidermis of Stage 11 at 7 days. Nerve fibres still present. High power.

FIG. 7. Portion of a sheet of presumptive epidermis cells 6 days after a 2-hour treatment with 0·1 M LiCl administered to explants made from Stage 11. Cytoplasm does not stain; pigment-rings and kidney-shaped nuclei are visible. Low power.

FIG. 8. Mesenchyme-like cells in a 6-day culture of presumptive epidermis in solution *X* containing 0·005 M LiCl. Low power.

PLATE 3

FIG. 9. High-power view of some cells at edge of sheet of 'pigment-ring cells' after 10-day culture. Stage 11 presumptive epidermis, source of the cells, was treated with 0·005 M LiCl in solution *X* for 18·5 hours.

FIG. 10. Early stage in formation of melanophores from presumptive epidermis 15 days after an 18·5-hour treatment with 0·005 M LiCl of explants from Stage 11. High power.

FIG. 11. Vacuolization of pigmented cells in process of becoming highly dendritic melanophore. Stage 11 presumptive epidermis was treated in 0·1 M LiCl for 4·5 hours. High power.

FIG. 12. Notochord from presumptive epidermis treated at Stage 11 with LiCl and CO_2 for 5 hours. Culture was fixed on eighth day. High power.

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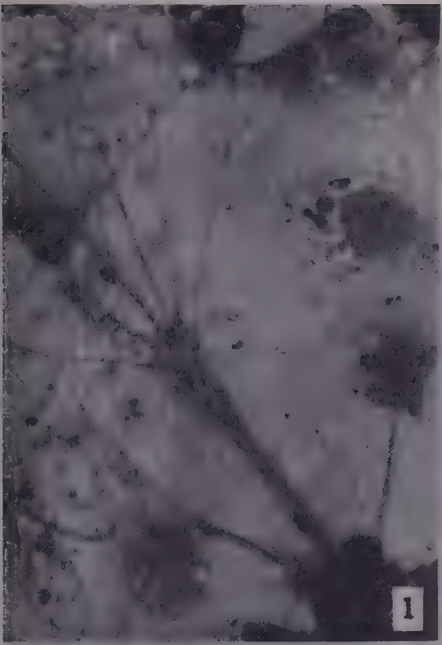

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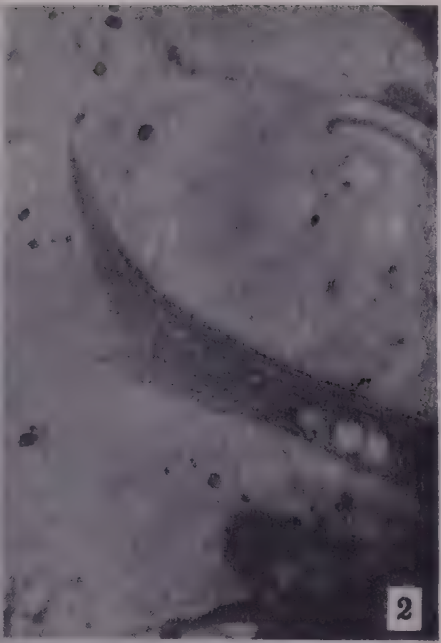

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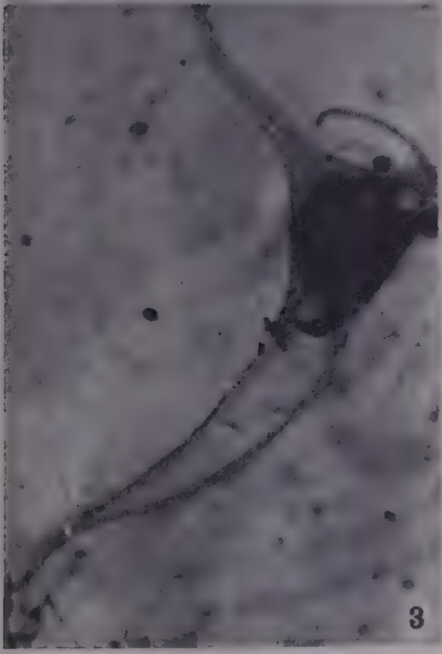

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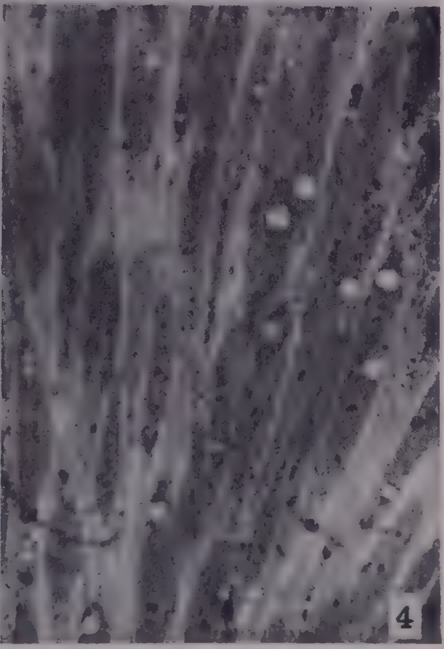

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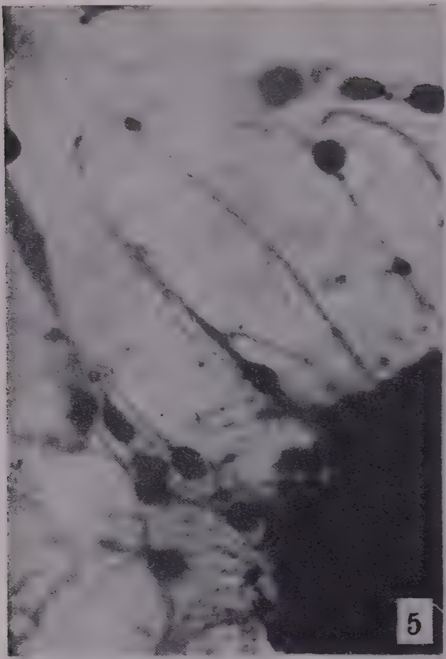
L. G. BARTH *and* L. J. BARTH

Plate 1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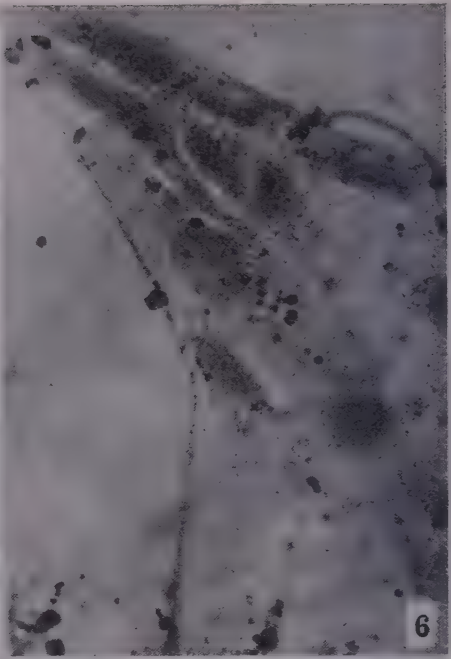
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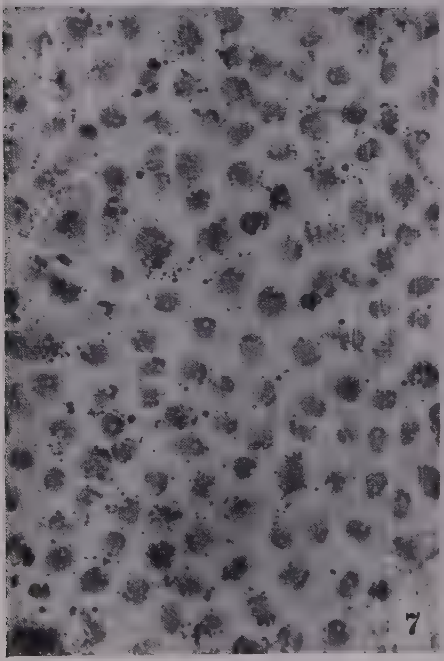
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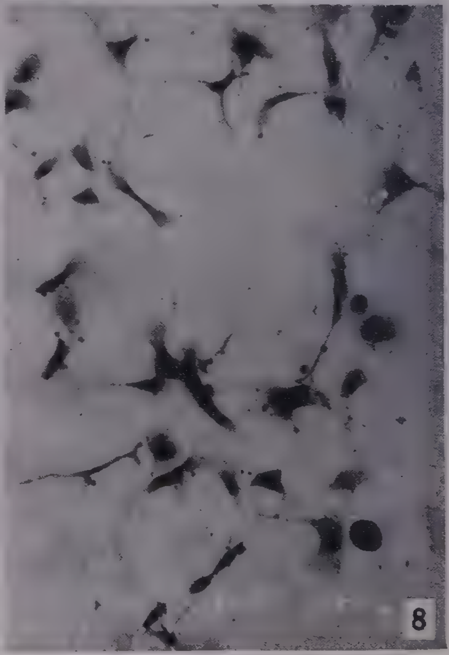
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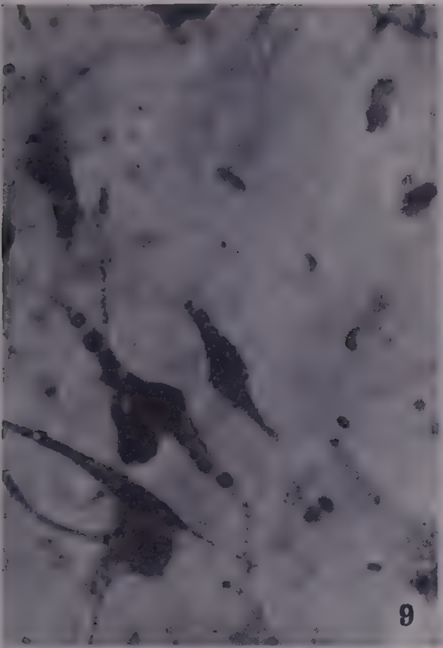
Plate 2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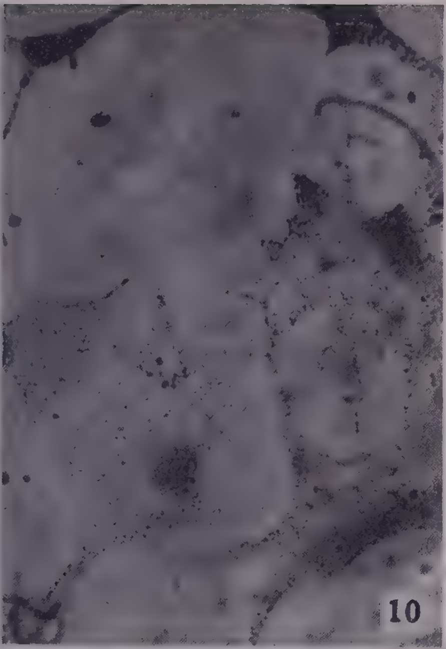
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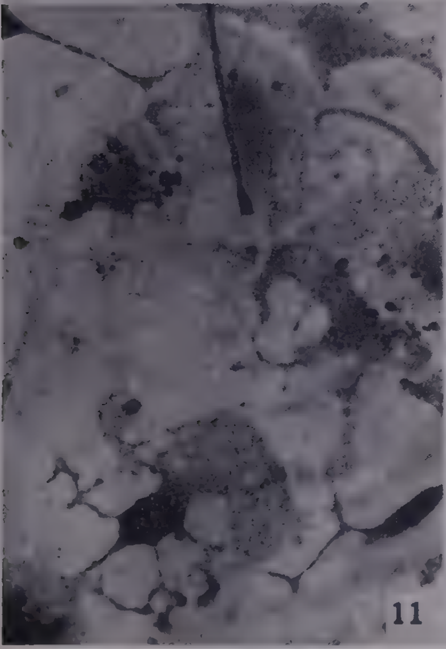
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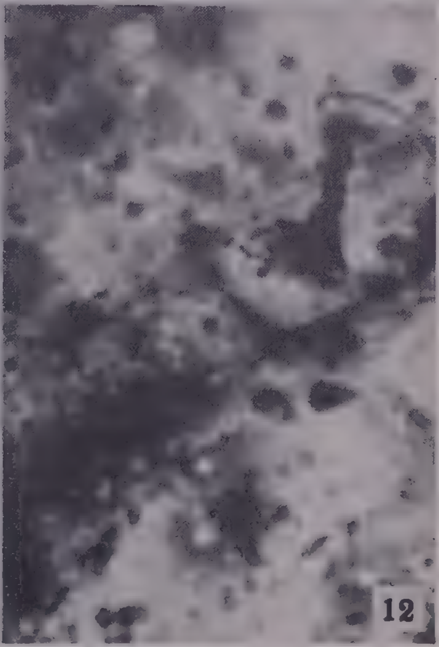
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Plate 3

Metachromasia Observed in the Living Eggs of *Arbacia punctulata* and its Cytochemical Analysis

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WITH ONE PLATE

INTRODUCTION

IT has long been known that metachromasia (a change that occurs in the absorption spectrum of certain basic dyes when placed under certain conditions) can be used as a valuable, though not specific, cytochemical method. The physical as well as the chemical conditions promoting metachromasia or affecting its intensity have been studied *in vitro* on microscopic sections of fixed tissues and, sometimes, on living material by numerous investigators (see Dalcq & Massart, 1952). From a chemical point of view it can be assumed that metachromasia is due to 'an electro-absorption of the cation of the basic dye by the acidic groupings of macromolecules, followed by an orientation of the organic skeleton of the dye by van der Waals forces' (Massart, 1953). Studying the metachromasia of living bacterial suspensions, Massart came to the conclusion that the substance responsible for it must be an acidic polysaccharide localized at the surface of the bacteria.

Using dilute solutions of toluidine blue, Dalcq (1952) was able to observe a strong purple metachromatic staining of certain granulations in a number of tissues and in the living egg of some mammals. His analysis emphasized the dependence of the phenomenon on certain factors such as light, temperature, pH, and dye concentration. Using their respective results, Dalcq & Massart (1952) showed that in this case, as in the bacterial suspension, acid mucopolysaccharides can be considered as responsible for the metachromasia of these cytoplasmic inclusions. As they point out, however, this vital staining has by itself no specific cytochemical value, but draws attention to certain constituents, the chemical composition of which can be subsequently diagnosed by appropriate techniques.

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In the case of the rodent egg (rat, mouse), the presence of mucopolysaccharides in the metachromatic granulations has been verified by cytochemical tests (Mulnard & Dalcq, 1955). In addition, acid phosphomonoesterase has been detected in the same inclusions (Mulnard, 1955), and it will be seen that in all species further investigated certain categories of cytoplasmic granulations can be defined by these three chemical properties: metachromasia, acid phosphatase activity, and the presence of acid mucopolysaccharides.

The eggs of the rat or mouse are cytologically ideal for this kind of investigation because of their transparency and small size. However, the mammal egg cannot give more than static information since development can be directly observed for only a short time, though the images that could be recorded suggested a dynamic evolution of the metachromatic structures. For this reason it was decided to shift the analysis to marine eggs, which should provide both suitable transparency and continuous observation of the development. Seven species of Invertebrates and Prochordates have been investigated so far: the Echinoderms *Paracentrotus lividus* and *Psammechinus miliaris* (Pasteels, 1955; Pasteels & Mulnard, 1957), the Molluscs *Barnea candida* and *Gryphaea angulata* (id.), the Annelid *Chaetopterus pergamentaceus* (Mulnard, 1958) and the Tunicata *Ascidiella aspersa* and *scabra* (Dalcq, 1957, 1959).

With some variations in details, and with certain complications in the special case of *Ascidiella*, all species have revealed the same fundamental features in the evolution of metachromatic phenomena in the living egg. This evolution can be outlined roughly as follows, taking as examples the eggs of *P. lividus* and *B. candida* which were the first to be studied. When stained for a short period in dilute solutions of toluidine blue (or brilliant Cresyl blue) in sea-water, and then placed back in normal unstained sea-water for observation, recently fertilized eggs exhibit a *primary* moderate (violet) metachromasia due to small granules (called α granules)[1] evenly distributed throughout the cytoplasm. At a definite time, variable with the species, a new type of larger granulation (called β granules) makes its appearance, immediately showing a strong (purple) metachromasia.

Experiments performed on *B. candida* have verified the fact that this new type of inclusion is not directly stainable by the dye solution. The β granules must receive the active group of the dye through the α granules, justifying the term of *secondary metachromasia*. Their appearance and evolution occur in close association with the nucleo-mitotic apparatus; as cleavage proceeds, they become distributed between the blastomeres and they increase in number at each prometaphase. Their metachromatic stainability is highly dependent on normal aerobic conditions and cinematographic records indicate that they display autonomous motility. The α and β granulations behave in totally different ways when centrifugal forces are applied to the eggs: the α granules are heavier than the β and they are found in the centrifugal zone in close association with the yolk. The β granules, on the contrary, are light elements and, when not adherent to the mitotic apparatus, concentrate in the top layer of the hyaline zone, often in contact with the oil drop. During mitotic activity, higher centrifugal forces are required to release them from the asters or spindle to which they are firmly bound. In the egg of *C. pergamentaceus* (Mulnard, 1958) a third metachromatic material, of granular nature, has been detected by means of centrifugation. It behaves as a direct precursor of the β granules, and its density and degree of metachromasia are intermediate between those of the α and the β granules. In *A. aspersa* also, additional metachromatic structures have been found and discussed by Dalcq (1957, 1959).

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[1] The granules called by our group α or β should not be confounded with the α, β, or γ inclusions described in the *Limnaea* egg (Raven, 1958, pp. 13 and 81).

Cytochemical tests have shown that in all cases (with the single exception of *P. lividus*) both α and β materials contain more or less acidic mucopolysaccharides and exhibit a high acid phosphatase activity. In *P. lividus* the α material lacks this high activity, whereas the β granules show it to the same extent as in the other species.

The present investigation of the egg of *Arbacia punctulata* must be considered as a part of a broad research programme. It adds new information to the results already obtained on the topics outlined. The analysis is not as extensive as Pasteels's on *P. lividus*. The lateness of the season and the relatively short time that could be devoted to the study account for the lack of some important information which we had hoped to obtain, particularly on the direct or indirect stainability of the β granules and the relationship between α and β granules. Nevertheless, our results are clear and complete enough to contribute in general to the principles acquired from the earlier observations on the metachromatic structures of the living egg.

MATERIAL AND METHODS

Sex determination was accomplished by employing the electrical method of Harvey (1953). An alternating current of 10 volts (from a step-down transformer) was passed through the animal by means of lead electrodes placed on the test until there was an extrusion of gametes from the gonopores. The few eggs or sperm released are easily distinguishable by their colour, the eggs being red and the sperm white. The animals were then washed clean of gametes by a quick bath in tap-water and were thus segregated by sex until used for experimentation.

The eggs were collected by injecting approximately 1 c.c. of 0·5 M KCl into the body-cavity. They were then immediately washed twice by decantation and used as a stock suspension. Sperm were obtained as a 'dry suspension' by removing the testes from the opened animal and placing them into a dry stender dish, which was kept in a refrigerator (0° C. (Harvey, 1956)).

To fertilize the eggs 2–4 c.c. of the dense stock suspension were diluted into

30 c.c. of filtered sea-water equilibrated to 20° C. One or two drops of the 'dry sperm' material were also diluted in 50 c.c. of sea-water and about two drops of this sperm preparation were mixed with the dilute egg suspension. The fertilized ova were then kept at 20° C. in order to give consistent rates of development from one experiment to another with the same egg stock. The egg stock was discarded if less than 80 per cent. of the eggs failed to develop a fertilization membrane within 10 minutes after insemination. The schedule of cleavage and development was similar to that given by Fry (1936) and Harvey (1956).

Five to ten minutes after fertilization the eggs were immersed in a 0·01 per cent. solution of toluidine blue (= TB) in sea-water where they were allowed to stay for 5 minutes. They were then thoroughly washed in several baths of normal sea-water, filtered 2–3 times through cheese-cloth to avoid agglutination such as may occur in eggs with overstained membranes. Finally, the eggs were placed in plain sea-water for the continuous observations.

Very critical observation of metachromasia in fertilized *Arbacia* eggs is somewhat difficult even after strong centrifugation because the numerous red pigment (echinochrome) bodies remain lodged in the firmly gelled cortex of the egg, obscuring to some extent whatever coloration may be present in the deeper cytoplasm. But if the eggs are subjected to a suitably calibrated pressure-centrifugation, a solation of the cortex occurs and the pigment bodies may be displaced completely from the centripetal hemisphere, or even to the extreme centrifugal region (see Marsland, 1950 and 1956). Consequently, the pressure-centrifuge technique was useful in some of the experiments.

The pressure-centrifuge (Brown, 1934) permits pressures up to 15,000 lb./in.^{2} to be sealed by a needle valve into one of the two centrifuge chambers, while the other, of identical radius, contains cells at atmospheric pressure. Thus the control and pressurized cells are simultaneously subjected to the same centrifugal force (up to 60,000 × gravity) and the temperature is controlled by placing the apparatus in a constant-temperature housing.

In the present experiments the stained eggs were centrifuged for 2 minutes at a pressure of 6,000 lb./in.^{2}, using a force of 41,000 × gravity, at 20° C. This pressure–force–temperature combination gave an excellent displacement of the pigment vacuoles and a distinct stratification of the other cell components. Moreover, it had little or no detrimental effect upon further development except that the first cleavage was delayed slightly (3–4 minutes).

Living centrifuged eggs were occasionally stained by a 1 per cent. solution of Janus green B for detection of mitochondria. This treatment is highly toxic, as other investigators have already pointed out. Although the eggs could not be kept in good condition for a long time, the period of observation possible was sufficient to locate the mitochondria and compare their distribution to that of the metachromatic granules.

The following cytochemical reactions were used to detect the acid phosphomonoesterase and the polysaccharides.

For *acid phosphatase activity* the technique was the same as that used in the case of *B. candida* and *G. angulata* (Pasteels & Mulnard, 1957) or *C. pergamentaceus* (Mulnard, 1958). It was applied to the eggs *in toto* after 2 hours' fixation with cold formol-saline (sea-water 9 parts, formalin 1 part) and 2 hours' washing. The substrate was a mixture of α and β glycerophosphate, at pH 5·3.

The incubation time did not exceed 6 hours at 37° C. The sites of activity were revealed as black PbS precipitate. Parallel controls were always run by omitting the substrate of the incubation mixture.

Polysaccharides were revealed on paraffin sections of eggs fixed in picric formalin (formalin 25 per cent. saturated with picric acid) by the PAS reaction (see Pearse, 1954) alone or after salivary digestion (active saliva, 3 vol.; dist. water, 2 vol.; 1 hour at 37° C.). Strongly acid mucopolysaccharides were more specifically stained with Alcian blue after the same fixation (Steedman, 1950; Adams & Sloper, 1955). Less acidic mucopolysaccharides were detected by the same staining technique following a strong oxidation by sulphuric permanganate (procedure of Cordier & Herlant, see Herlant, 1958).

Here are the details of the technique:

1. Alcian blue solution (pH 0·2): dissolve 1 g. of Alcian blue in 100 c.c. of 12 per cent. sulphuric acid. Boil for 1 hour, using a reflux-condenser. Cool and filter. Stain for 20 minutes, then wash thoroughly with distilled water (washing ensures differentiation).

2. Sulphuric permanganate

   Prepare stock solutions: *A* = 2·5 per cent. aqueous potassium permanganate.
   B = 5 per cent. sulphuric acid.

   Mix 1 vol. of *A*, 1 vol. of *B*, and 6 vol. of distilled water (this mixture must be freshly made).

   Oxidation takes 1–2 minutes in this solution. Then wash with distilled water and bleach by short treatment with 5 per cent. sodium metabisulphite. Wash again and stain with Alcian blue as previously described.

OBSERVATIONS ON VITAL METACHROMASIA

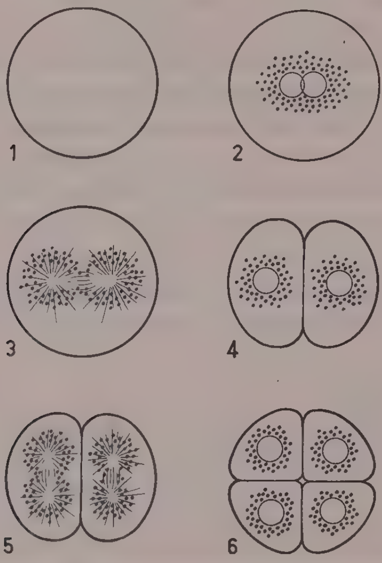
The short treatment with a rather concentrated TB solution (0·01 per cent.) did not seem to affect the vitality of the eggs. Apparently there is a specific variation of susceptibility to the dye. Smaller concentrations of TB inhibit cleavage in the egg of *Chaetopterus*, whereas even the developmental timing is not affected by the staining in *Arbacia*.

Continuous observation did not go beyond stages IV and VIII, although the stained eggs were always kept overnight to check the normality of further development.

The results will be separated into two groups dealing, respectively, with normal and pressurized-centrifuged eggs.

A. *Normal eggs* (Text-figs. 1–6)

The appearance of the egg stained 5 minutes after fertilization is constant (Text-fig. 1): the whole cytoplasm shows a diffuse and slightly metachromatic staining. In the clearer nuclear region the colour is a pure, light violet, whereas in the rest of the egg it is combined with the fundamental yellow tint of the cytoplasm to give a greyish-violet appearance. The dye is also taken up by the pigment bodies which are mainly concentrated in the cortical layer. It is difficult to decide whether these pigment bodies are metachromatic or not. Their final deep violet, almost black, staining results from a combination of their natural bright red colour with the acquired (blue or violet?) one. What one can say is that the metachromasia, if present, must be of the weak type. Otherwise the final colour should be a red one (association of brick-red and purple).



TEXT-FIGS. 1–6. Schematic drawings representing the evolution of the metachromatic β granules in the early development of the normal fertilized *Arbacia* egg, as seen *in vivo*. The egg has been stained briefly, 5 minutes after fertilization, in a 0·01 per cent. solution of toluidine blue. (The pigment bodies have been omitted, and the size of the β granules has been exaggerated.)

1. Before the paired pronuclei stage. No granules visible.
2. Stage of the paired pronuclei: appearance of the first purple granules around the pronuclei.
3. Metaphase of the 1st cleavage mitosis. Numerous β granules distributed in the asters and somewhat along the spindle.
4. Resting stage II. β granules gathered around the nuclei.
5. 2*d* cleavage mitosis. Metaphase. New increase of the amount of β granules. Same distribution as during first metaphase.
6. Resting stage IV. Perinuclear localization of the β granules.

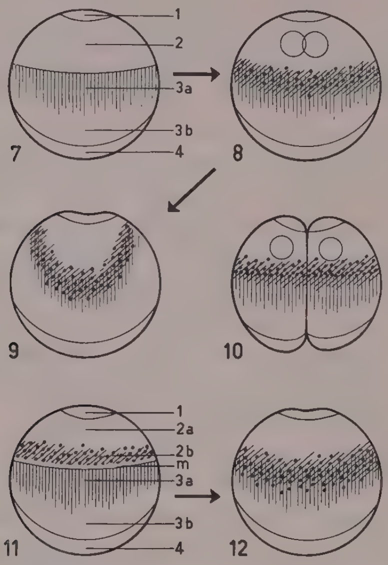
This condition remains unchanged until the advanced pronuclei stage. At that time a change in colour occurs in the area immediately surrounding the nuclear zone: a purplish cloud becomes visible in that zone. No structure can at first be detected in this purplish area, but soon afterwards, at about the time of the prophase of 1st division, it gradually becomes invaded by small, deep purple-stained granules (Text-fig. 2), the number of which increases at a high rate during metaphase. In the latter period the granules have concentrated in the asters and at the surface of the spindle, completely surrounding the mitotic apparatus (Text-fig. 3). Their subsequent evolution is entirely comparable to that of the β granules described in the species previously studied (*Paracentrotus*, *Barnea*). Mitosis divides the whole set equally between the first blastomeres. At stage II the granules surround the resting nucleus, and their number does not noticeably change (Text-fig. 4). At pro- and metaphase of the 2nd division they gather in the asters and along the spindle (Text-fig. 5) and obviously their number rises again. They are equally divided among the first four blastomeres, where they take a perinuclear position at the resting stage (Text-fig. 6). The same cyclic evolution can be observed during the 3rd and further divisions. No attempt has been made to observe an eventual segregation of the β granulations into definite groups of cells, all efforts being concentrated on the problem of the physical and chemical properties of the metachromatic granules.

B. *Pressurized-centrifuged eggs* (Text-figs. 7–12)

The results of this treatment, applied to previously stained eggs, vary with the developmental stage.

When the fertilized egg is treated before the pronuclei stage, at a period during which no β granules are detectable, and observed immediately after treatment, four of the layers described by Harvey & Lavin are recognizable (Text-fig. 7): from the centripetal to the centrifugal pole (1) the lipidic mass, (2) the clear (or hyaline) zone, (3) the dark (or vitelline) zone, and (4) the pigment bodies crescent. The first two layers are entirely unstained. The fourth layer, composed of packed pigment bodies, is dark violet, almost black. The dark, or vitelline zone, appears heterogeneous as to its staining properties. The upper part contains a light-violet band (3*a*) that extends up to the clear-cut limit of the hyaline zone, whereas in the opposite direction it gradually fades, leaving the lower half of the vitelline zone unstained (3*b*). A similar stratification is observed in the unfertilized egg submitted to a 35,000 g. centrifugation (the viscosity of the unfertilized egg is lower and pressurization is not required). Thus, except for a part that possibly might be in the pigment crescent—which is not likely—the whole of the diffuse material (α granules) bearing the slight primary metachromasia during the first period of normal development is concentrated by centrifugation in the upper half of the vitelline area.

The staining and pressure-centrifuge treatment do not prevent the egg from proceeding with its normal early development. The pronuclei soon become visible and pair in the hyaline zone. In the meantime an important modification occurs at the boundary between the clear and dark zones. This boundary loses its sharpness, while the violet band (3*a*) gradually brightens—its colour changing to a more purplish one—enlarges and invades the lowest part of the hyaline zone. A transitory situation is realized in which a purplish cloud with fading



TEXT-FIGS. 7–12. Schematic drawings of the behaviour of the metachromatic structures in living pressure-centrifuged *Arbacia* eggs observed *in vivo*.

7. Stratification observed immediately after treatment of an egg before the stage of the paired pronuclei. 1, oil drop; 2, hyaline zone; 3, vitelline zone; 4, pigment bodies. Metachromasia due to α granules concentrated in the upper half of the vitelline zone (3*a*—vertical strips).

8. The same egg at the stage of the paired pronuclei. Appearance of the purplish cloud (oblique lines = *X* material) and formation of numerous β granules in this area.

9. The same egg at metaphase of first mitotic cleavage.

10. Stratification obtained by pressure-centrifugation at stage II. β granules concentrated in the lower hyaline and the upper vitelline layers with the third material.

11. Stratification obtained by treatment at the beginning of the first mitotic cleavage. Third material and preformed β granules concentrated in the lower hyaline layer (2*b*), and separated from the α material (in 3*a*) by the mitochondrial band (*m*).

12. The same egg after 10–15 minutes. Same appearance as in the preceding experiment (Text-fig. 8). (For detailed explanation see text.)

outlines and delicate granular structure occupies the adjacent portions of the clear and the dark zones. Soon afterwards light purple inclusions, having all the characteristics of the β granules, make their appearance in that material. Their number increases rapidly so as to crowd the whole area of the purplish cloud (Text-fig. 8). When the first mitotic figure has become established in the hyaline zone, the granular area often bends and half-encircles the mitotic figure (Text-fig. 9). Many of the β granules move upwards and come into contact with the centrifugal side of the asters and spindle. First cleavage occurs in the centrifugation axis, dividing the set of granules between the first two blastomeres as in the normal egg. At stage II the redistribution of the stratified materials continues to such an extent that the 2nd division appears almost normal and the β granules are typically divided among the first four blastomeres.

When the stained eggs are pressurized and centrifuged at a later stage, e.g. at the paired pronuclei stage, the layers are distributed in the same sequence as in the preceding experiment, but the hyaline zone now appears to be heterogeneous, its lower half being occupied by a diffuse purplish band in which a variable number of preformed β granules is scattered (Text-fig. 11, zone 2*b*). The violet band of the α material occupies the typical position in the upper half of the vitelline zone (zone 3*a*).

This condition can be observed for only a very short time. Nevertheless, careful observation permits the following statements: (1) The diffuse, purplish background material of the band 2*b* cannot be confused with the metachromatic α material present in the band 3*a*. When observed with high magnification it reveals very tiny granules. (2) When the preformed β granules are in sufficiently large amount, there always exists a clear intermediate band immediately adjacent to the dark zone and devoid of β granules. Its position indicates that it corresponds to the mitochondrial band of Harvey (*m* in Text-fig. 11). This conclusion is corroborated by the use of Janus green B which stains electively band *m* but not band 2*b*.

In the few minutes following the treatment, a rapid change occurs in this region of the egg. New purple β granules appear all over band 2*b*. In the meantime the limit of band 3*a* loses its sharpness, exactly as in the preceding experiment. The β granular area enlarges, the clear mitochondrial zone being no longer visible. Soon the egg takes the same appearance as one that had been centrifuged at an earlier stage (Text-fig. 12), and subsequent development is the same in both cases. Pressure-centrifugation performed at a still later stage leads to a less clear-cut stratification. At stage II (Text-fig. 10), the band 3*a* is still recognizable but the band 2*b* is not as well defined as earlier: the purplish cloud is narrower and lies close to the dark zone into which it seems to penetrate. The mitochondrial band is no longer distinguishable and numerous β granules are scattered not only in 2*b* but also in the upper part of 3*a*.

CYTOCHEMICAL OBSERVATIONS (Figs. A, B, and C of Plate)

When Gomori's technique for *acid phosphomonoesterase* is applied to the whole normal egg, the cytoplasm develops a uniformly dark coloration. Even with very short incubation times (15–30 minutes) no valuable information can be obtained as to the fine distribution of the enzymatic activity, since the reaction gives the whole cytoplasm a diffuse brown colour. When, on the contrary, the reaction is applied to centrifuged, or pressurized-centrifuged eggs, satisfactory results can be attained.

Centrifuged unfertilized eggs give a very simple and constant image after a 6 hours' incubation time: the whole of the dark zone, including layers 3 and 4 (yolk and pigment bodies) is homogeneously blackened, and controls show that this corresponds to a true enzymatic activity. No other positive structure can be detected in the rest of the egg.

The reaction gives a different result when applied to a pressurized-centrifuged fertilized egg (Plate, fig. C). Here, in addition to the blackening of zones 3 and 4, already observed in the unfertilized egg, a strongly positive band appears in the clear zone. Its width and position indicate that it obviously corresponds to band 2*b* (lower half of the hyaline zone) in which the purple cloud and the granules have been located in the living egg stained with toluidine blue. Despite the local diffusion, always important at the limits of such strongly positive structures, a clear negative band always lies between the two positive regions. Careful comparisons of treated eggs and blank controls show that this negative zone undoubtedly corresponds to the mitochondrial band. Layers 1 (lipidic) and 2*a* (upper hyaline) remain entirely negative. The very strong staining of band 2*b* does not permit a good definition of the structures responsible for the reaction, but the use of shorter incubation periods shows a brown diffuse background containing scattered black granules. The short-incubation-time technique also gives interesting information about the reaction in zones 3 and 4. After a 15–30-minute incubation the reaction has its maximum intensity in the upper half of zone 3 (3*a*) and in zone 4, with a band of weaker positivity (3*b*) in between.

The sites of highest acid phosphatase activity thus closely correspond to the layers in which the metachromatic granules have been stratified.

The same satisfactory relationship can be observed when reactions for *mucopolysaccharides* are studied.

On normal eggs the PAS reaction is not useful since it stains several kinds of cytoplasmic constituents, including the yolk elements. The result is a totally stained purple egg, with no discernible detail. Even salivary digestion does not clear the cytoplasm, as the main glucidic component of the yolk appears not to be glycogen. Even on centrifuged eggs the PAS reaction is of little value. Zones 3 and 4 are both heavily stained and the staining extends into the mitochondrial band and zone 2*b*. However, the staining is so intense that no details can be observed.

The Alcian blue technique, on the other hand, brings out some highly significant information. When used alone on normal eggs, Alcian blue almost selectively stains the β granulations and the background cytoplasm corresponding to the purple cloud observed *in vivo* with toluidine blue. The rest of the cytoplasm is pale blue. When used after a strong oxidation by sulphuric permanganate, Alcian blue gives the same appearance except that the diffuse cytoplasmic staining is reinforced and the pigment granules are deeply stained.

Pressurized-centrifuged fertilized eggs, submitted to Alcian blue alone, take the appearance shown by fig. A of the plate: zones 1, 3 and 4 are negative; zone

2*a* is pale to fairly intense blue; zone 2*b* shows deeply stained granulations with a clear blue background.

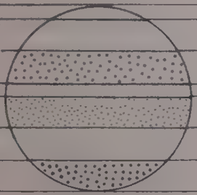
After oxidation Alcian blue gives the result shown by fig. B of the Plate. The fertilization membrane is now stained, but there is no change in zones 1 and 2*a*. Zone 4 (pigment bodies) is positive. The staining of the granules in band 2*b* is reinforced. But the main change occurs in zone 3. The upper part of it (3*a*) is now deeply stained. The staining has a maximum intensity in a narrow band close to the clear-cut limit of the zone and gradually fades in the centrifugal direction, leaving 3*b* unstained. Sometimes bands of stained material run parallel to the centrifugation axis (indicated by arrow on fig. B of the plate), showing that the α material is not completely sedimented at the speed (41,000 g.) utilized in the experiment.

There is always a clear unstained split, corresponding to the mitochondrial band (*m*), between 2*b* and 3*a*.

Thus here again, with the exception of some material sedimented in zone 2*a*, the localization of mucopolysaccharides closely corresponds to the layers into which the metachromatic structures have been stratified. But this technique also points out a striking difference between β and α materials: β granules (in 2*b*) contain strongly acidic mucopolysaccharides (directly stained by Alcian blue), whereas α granules (in 3*a*) contain less acidic mucopolysaccharides (stained by Alcian blue only after liberation of acidic groups by strong oxidation).

SUMMARY OF THE OBSERVATIONS ON PRESSURIZED-CENTRIFUGED EGGS

Text-fig. 13 summarizes the results obtained on the *Arbacia* egg when it is pressure-centrifuged at the advanced pronuclei stage. It shows the layers and sub-layers in which the metachromatic constituents of the cytoplasm have been stratified, owing to their relative density. It emphasizes the close correspondence that exists in those layers between metachromasia and the occurrence of muco-

	layer		sub-layer	metachromatic structures	metachromasia	cytochemistry
1	lipidic			—	no	—
2	clear or hyaline		2a	—	no	strongly acid mucopolysaccharides ± (RNA+)
			2b	β granules & X material	strong fair	strongly acid mucopolysaccharides ++ acid phosphatase ++ (RNA+)
m	mitochondrial			—		(RNA ++)
3	dark or vitelline		3a	α granules	slight	weakly acid mucopolysaccharides ++ acid phosphatase ++
			3b	—	no	acid phosphatase +
4	pigment			pigment gran. ?	weak	weakly acid mucopolysaccharides ++ acid phosphatase ++

TEXT-FIG. 13. Diagrammatic representation of a pressurized *Arbacia* egg centrifuged at the beginning of the first mitotic cleavage, indicating the occurrence of the metachromatic structures and of some cytochemical properties in the layers and sub-layers, and showing the correspondence between the sites of metachromasia and those of mucopolysaccharides and acid phosphatase activity.

polysaccharides and acid phosphatase activity. The question mark in zone 4 indicates the possibility that a metachromatic material may be stratified in that zone. If so, it is obscured by the accumulation of closely packed pigment bodies. In addition, the stratification of the RNA-containing structures has been indicated as it appears in sections treated by the Unna-Brachet technique (methyl green-pyronine). It shows that the whole of the RNA is localized in the hyaline zone and in the mitochondrial band. The latter result is in accordance with Pasteels's observations on *P. lividus*. It is difficult to decide whether RNA is localized in the mitochondria themselves or in the background material.

DISCUSSION AND CONCLUSIONS

The study of the early development of *Arbacia punctulata* by means of vital staining with toluidine blue and by some cytochemical methods has shown that this species, like the ones previously studied, possesses a metachromatic structural (granular) equipment having well-defined physical and chemical properties and a characteristic behaviour. A convenient terminology has been adopted for the various constituents of the equipment. This is purposely non-specific (α and β granules, material *X*), because the true nature of the material is still unknown. The terminology refers only to basic properties, and from that point of view it can be applied to the metachromatic inclusions of *Arbacia*.

A diffuse, finely granular cytoplasmic material, present initially in the unfertilized egg, and exhibiting a slight primary metachromasia is referred to as α granules. This fundamental equipment of the egg-cell has been observed in all species which have been studied. It reveals several basic, as well as some variable, properties. The α granules in all cases are directly stainable. That the diffuse character of the primary metachromasia is due to a granular constituent and not to the background cytoplasm itself is well demonstrated by centrifugation experiments in which, in every case, the α material is stratified with the yolk in the centrifugal half of the egg. Within these limits the relative density of the α granules may vary from one species to another. In *B. candida* (Pasteels, 1955; Pasteels & Mulnard, 1957) and *P. lividus* (Pasteels, 1958) they are sedimented in the lowest region of the vitelline layer and represent, in *Barnea*, the heaviest elements of the cytoplasm, whereas, in *Paracentrotus*, only the free mitochondria are heavier. In *Ascidiella aspersa* and *scabra* (Dalcq, 1957, 1959), the α granules are widely distributed in the subcortical layer of the unfertilized egg. After fertilization, as maturation proceeds and while the yellow plasma is concentrating at the vegetative pole, most of the α granules move toward the same pole and accumulate in the cytoplasmic region immediately adjacent to the yellow plasma; in that region a diffuse metachromatic material can also be seen in addition to the α granules. At the metaphase of the first mitotic cleavage, many of the metachromatic granulations invade the asters, some of them being smaller and exhibiting the properties of β granules (i.e. they segregate by centrifugation into the hyaline zone while the α granules concentrate at the upper limit of the yolk). In *C. pergamentaceus* (Mulnard, 1958), after centrifugation, the granules are concentrated in the upper layer of the vitelline zone. The same behaviour is observed in *Arbacia punctulata.* In Echinoderms, Molluscs, and worms, the α granules have been shown to contain acid mucopolysaccharides. In *Arbacia* the use of the Alcian-blue technique has thrown some more light on the nature of the glucidic constituents of the metachromatic elements. It has been shown that the α granules contain weakly acidic mucopolysaccharides since they are stained only after strong oxidation. On the contrary, the β granulations are directly stained by Alcian blue and preliminary oxidation does not much reinforce this staining. The same observation has been made in *P. lividus* (Pasteels, 1958). As a matter of fact, this is the first indication of a chemical difference between α and β granules.

Except in one species, *P. lividus* (Pasteels, 1958), the α granules always show an important acid phosphatase activity. In *Arbacia* this is probably the case, although the strong positivity of the whole vitelline zone does not make it particularly clear after long incubation of centrifuged eggs. But after shorter incubation, the reaction fades in the lower half of the yolk (3*b*) leaving the upper half (3*a*) -in which the α granules are concentrated—strongly positive. Such a qualitative difference between the yolk and the α elements does not exist in *Paracentrotus.* In this species an incubation period leaving the yolk unstained also prevents any reaction of the α granules, whereas the β granules are still positive. Thus it is possible that the negativity of the α granules in *Paracentrotus* is only an apparent one.

The β granules differ markedly from the α in several properties. The β granules are larger, though there can be important variations of size from one species to another and even within a single set of granules. They are lighter and always stratify in a more centripetal layer after centrifugation. Here also variations in relative density can be observed.

Generally the β granules, when not linked or only weakly linked to the mitotic apparatus, gather in the hyaline zone where their final position may vary from the upper layer, at the limit of the oil drop (*B. candida*, *G. angulata*, *Ascidiella*), to the lower layer, at the limit of the yolk or mitochondrial band, as is the case in *Arbacia punctulata.* Various intermediate positions are observed, as in *C. pergamentaceus.* Only in *P. lividus* do the β granules stratify in the upper vitelline layer. In all species they show close relations to the nucleo-mitotic apparatus, and this constant property is verified in *Arbacia.* Here they are localized around the nuclei during the resting stage, and during mitosis they are found along the spindle and in the asters firmly bound to the fibres. This is demonstrated by the fact that only high centrifugation forces can remove them from their position, and still never completely. Not only do the β granules show spatial relations to the mitotic apparatus, but also their formation seems to be dependent on active mitotic structures: in *B. candida* their first appearance can be observed *in vivo,* in the spermaster, and, to a lesser extent, in the astral zone of the 2nd maturation division. In *Chaetopterus* they are even more precocious. Their observable formation takes place before fertilization, in the astral region of the first maturation division. In *P. lividus* (Pasteels, 1958) the β granules can be seen for the first time at the prophase of the first mitotic cleavage, and such is also the case in *A. punctulata*. It must be emphasized that these observations deal with granules appearing in the living egg stained with metachromatic dye and that this apparent time of formation does not necessarily correspond to the real one. In every case the β granules are characterized by their *indirect metachromatic stainability*: they are not directly stained by the dye solution. It has been clearly demonstrated that their metachromasia is dependent on a preliminary staining of the α granules and on the transfer of the active part of the dye from the latter to the β granules. Furthermore, the staining is reversible. Under anaerobic conditions the β granules lose their colour by release of the dye, as is demonstrated by an overstaining of neighbouring eggs that are still in an aerobic environment. Thus, when an egg is stained by the usual procedure, the pre-existent β granules are not demonstrated, and only those formed after the staining can be observed subsequently. In the case of *P. lividus*, Pasteels (1958) has emphasized this point, showing that, although the formation of metachromatic β granules is not observed *in vivo* until the beginning of the first mitotic cleavage, β granules do exist from fertilization on, since they can be detected at any stage after fertilization by the cytochemical tests for acid phosphatase and mucopolysaccharides applied to centrifuged eggs. The same observation can be made in *A. punctulata*, where the β granules are detected in the lower hyaline band (2*b*) of fixed centrifuged eggs by these cytochemical tests.

This raises the problem of the origin of the β granules. We have seen that at least in one of the studied species (*Chaetopterus*), their first formation does not depend on fertilization, since they appear in the deep aster of the first maturation division in unfertilized eggs as well. In the sea-urchin egg, for technical reasons, no information has been obtained concerning an early formation of β granules before fertilization during the maturation mitosis. It should be emphasized, however, that in *Arbacia* the cytochemical tests for acid phosphatase and mucopolysaccharides fail to demonstrate any β granules in the mature unfertilized egg. But these granules can be detected soon after fertilization, and thus it is probable that their initial formation takes place in the spermaster, as in *Barnea*.

This study on *Arbacia* has also verified the fact that a new set of β granulations is formed at each mitotic cycle. No experiment was attempted to confirm the results obtained in *Barnea* and *Paracentrotus* as to the transfer of the active part of the dye from the α to the β granulations. It can only be said that no likely source of dye, other than from the α granules, would seem to be available, since transfer from the pigment bodies seems most improbable.

Little can be said about the third component (*X* material) of the 'purplish cloud'. Dalcq (1957) has pointed out such a metachromatic material in *Ascidiella*. It has been demonstrated by means of centrifugation in *C. pergamentaceus*

(Mulnard, 1958). In *Arbacia* and *Chaetopterus* its properties and behaviour are very similar. The material is not detectable before fertilization, and it always has a fine granular structure. Its metachromasia is somewhat stronger than that of the α granules, and it also contains acid phosphatase and mucopolysaccharides. Its relative density is lower than that of the α granules. In *Chaetopterus* this density varies, the material being concentrated by centrifugation either in the upper or in the lower hyaline zone. In *Arbacia*, as well as in *Chaetopterus*, the β granules are formed in the centrifuged egg, exclusively in the area of the third material.

Pasteels's observations on centrifuged eggs of *Paracentrotus* are also in favour of the existence of such a third material. Up to the stage of the first mitotic cleavage, two layers of metachromatic granulations are found in the vitelline zone: (1) the α granules packed against the mitochondrial layer in the lowest vitelline region, (2) the β granules in the top layer of the yolk. After that stage an additional metachromatic material can be detected in the intermediate zone of the yolk, its position varying in height and width.

The significance of the third material has been discussed in the case of the *Chaetopterus* egg. Its behaviour and properties in *Arbacia* confirm the impression that it could be a direct precursor for the formation of the β granules, but the available evidence does not throw any light upon its relationship to the α granules.

It is too early to devote a long discussion to the problem of the nature of the metachromatic inclusions. We cannot yet specify the granular system to which they may belong. The answer to this question must await the accumulation of more information, including electron-microscope data obtained from many species.

In the early stages of this research the β granules were considered to be mitochondria. Later, as information accumulated, this opinion had to be changed. The first consistent argument against the mitochondrial nature of the β granules was obtained on *Paracentrotus*. An electron-microscope study of centrifuged eggs (Pasteels, Castiaux, & Vandermeerssche, 1958) showed the mitochondria divided by centrifugal force into two sets. The first one is in the oil cap and consists of lipid-linked mitochondria and the second is sedimented into the most centrifugal part of the egg, below the yolk. No mitochondria can be detected in any other layer, and particularly not in the β granular layer which lies on top of the yolk. Such a disassociation of mitochondria and β granules by centrifugation is observed in uncleaved *Arbacia* eggs. The β granules accumulate in band 2*b*, above the mitochondrial layer. The use of Janus green B and the reaction for acid phosphatase verify the sharp separation of those two layers; Janus green B stains vitally the mitochondria and not the β granules; acid phosphatase activity is found in the latter, not in the mitochondria (cf. Plate, fig. C).

In *Paracentrotus*, Golgi complexes are found in the same layer as the β granules, and this observation may indicate a basic relationship. The Golgi elements are now better known, since some have been isolated and submitted to chemical analysis (Worley, 1951; Schneider & Kuff, 1954; Dalton & Felix, 1954). Golgi elements have many points in common with the β granules, especially as to their vital staining properties, their PAS positivity, their acid phosphatase activity, and even their structural appearance. Yet there are discrepancies such as alkaline phosphatase activity which has not been observed so far in the β granules. Also β granules have much in common with certain inclusions described in cells cultivated *in vitro* (neutral-red inclusions of chicken fibroblasts and amoebocytes of *Helix*, see Crawford, 1957; and PAS-positive granulations of myogenic cells, Moscona, 1955).

We have even less information about the nature of the α granules, which also have been considered as very small mitochondria. It seems possible that they may be related to the lysosomes of de Duve *et al.* (1955) and it is interesting to note that in the opinion of certain investigators, such as Novikoff (1959), the lysosomes may be regarded as Golgi complexes. The same uncertainty exists concerning the nature of the third material.

Further investigations are needed, especially electron-microscope studies of normal and centrifuged eggs and isolation *in vitro* for biochemical analysis. This programme has already been initiated in the case of *P. lividus*. It will be continued on the most favourable species with the final purpose of defining the exact significance and physiological importance of the metachromatic structural equipment of the egg-cell.

SUMMARY

The analysis of the egg of *Arbacia punctulata* by means of vital staining with toluidine blue confirms the results obtained on other species. It shows a *primary metachromasia* due to tiny α granules scattered throughout the cytoplasm, and the appearance, at the time of the paired pronuclei, of *secondary metachromatic* β granules in close relation to the nucleo-mitotic apparatus.

High-speed pressure-centrifugation (Marsland's procedure) shows that the α granules are sedimented in the upper half of the vitelline zone, the β granules in the lower half of the hyaline zone. These two metachromatic layers remain separated by the mitochondrial band. A third granular, metachromatic material can be detected which may be a precursor of the β granules.

Cytochemical tests verify the presence of acid phosphatase in all the metachromatic structures and show that all contain mucopolysaccharides, which account for the metachromasia. The Alcian-blue technique reveals that the β granules contain strongly acidic mucopolysaccharides, whereas the α granules contain less acidic mucopolysaccharides.

RÉSUMÉ

L'analyse de l'œuf d'*Arbacia punctulata* au moyen de la coloration vitale par le bleu de toluidine confirme les résultats obtenus chez d'autres espèces. Elle

montre une *métachromasie primaire* due à de fins granules α éparpillés dans le cytoplasme, et l'apparition, au stade des pronucléus accouplés, de granules β à *métachromasie secondaire*, en relation étroite avec l'appareil nucléo-mitotique. Une pressurisation combinée à une centrifugation à haute vitesse (technique de Marsland) montre que les granules α se sédimentent dans la moitié supérieure de la zone vitelline, les granules β dans la moitié inférieure de la zone hyaline, ces deux couches métachromatiques restant séparées par la bande mitochondriale. Un $3^{\text{ème}}$ matériel métachromatique de structure granuleuse peut être détecté et se comporte comme un précurseur des granules β.

Des tests cytochimiques vérifient la présence, dans ces structures métachromatiques, de phosphatase acide et de mucopolysaccharides, ces derniers responsables de la métachromasie. La technique du bleu Alcian révèle que les granules β contiennent des mucopolysaccharides très acides, tandis que les α contiennent des mucopolysaccharides peu acides.

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EXPLANATION OF PLATE

Photographs of sections of *Arbacia* eggs treated at the pronuclei stage by two cytochemical techniques.

FIG. A. Fixation: picric-formalin. Paraffin embedding. Sections 7 μ. Alcian-blue technique. Selective staining of the β granules concentrated in band 2*b* (lower hyaline).

FIG. B. Same technique, but the Alcian-blue staining was applied after oxidation by sulphuric permanganate. In addition to the β granules, Alcian blue now stains the α material concentrated in band 3*a* (upper vitelline), but not thoroughly sedimented (back-left material indicated by arrow). The β granules and the α granules are separated by the unstained mitochondrial band (*m*). Strong staining of the pigment bodies crescent.

FIG. C. Fixation: cold formol-saline. Treatment *in toto* by modified Gomori's technique for acid phosphatase. Incubation time: 6 hours. Strong reaction of the whole of the vitelline and pigment zones and on band 2*b* (third material + β granules). Mitochondrial band (*m*), upper hyaline (2*a*), and lipidic (1) zones completely negative (see detailed explanation in text).

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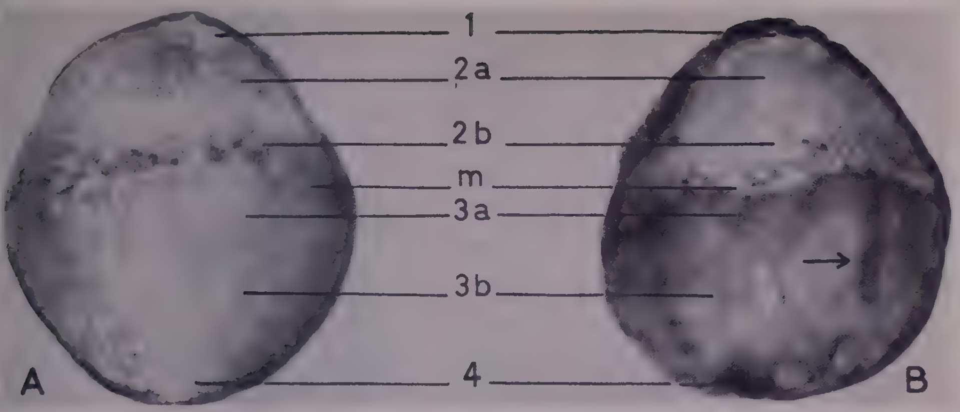

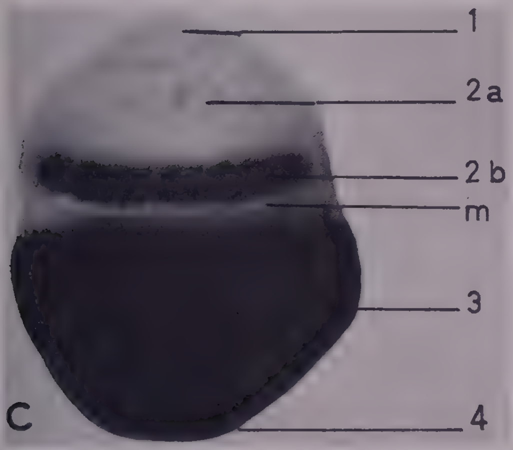

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Morphogenetic Events in Normal and Synchronously Dividing *Tetrahymena*

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WITH ONE PLATE

INTRODUCTION

SYNCHRONOUS cell-division has been induced in mass cultures of the small ciliated protozoan *Tetrahymena pyriformis* (Scherbaum & Zeuthen, 1954). While it is known that cells grow in a characteristic way during the synchronizing treatment the effect on the morphogenetic events associated with the cell cycle is not clear. Studies in ciliate morphogenesis generally have established the central position of the ciliary basal body, or kinetosome, in developmental processes. The kinetosomes are believed to be self-duplicating structures, the kinetosomal population of a daughter cell arising directly by kinetosomal reproduction in the parent cell. The species-specific pattern of the ectoplasmic cortex is largely a matter of the distribution of kinetosomes. Further, the kinetosomes appear to function either as building blocks or 'local organizers' in most, if not all, structural syntheses occurring in the cortex, i.e. in the production cilia, cirri, membranelles, trichocysts, and other ciliate structures (see Weisz, 1954). Two conflicting notes are available concerning the behaviour of kinetosomes and kinetosomal derivatives in synchronized cultures of *Tetrahymena*. Child (1957) has reported that kinetosome reproduction continues and new mouth parts are formed in cultures of *T. pyriformis* W subjected to the synchronizing treatment. On the other hand, Holz, Scherbaum, & Williams (1957) have reported that formation of mouth parts and also mitosis is inhibited by the synchronizing treatment in *T. pyriformis* WH-6 (mating type I, variety 1). In the present study kinetosomes and kinetosomal derivatives in *T. pyriformis* GL were studied in detail in order to elucidate the relation of the morphogenetic events of the cell cycle to the experimentally induced synchronous cell-division.

On an earlier occasion cell-growth in synchronized cultures was studied (Scherbaum, 1956). The mean cell size was found to increase from three- to fourfold as a result of the treatment. When the cells were grouped in logarithmic size

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classes and analysed with the probit method it was found that, on a percentage basis, the small cells grew less than the large cells during treatment. Preliminary observations of the nuclei of synchronized cells have indicated an absolute increase in nuclear size (Zeuthen & Scherbaum, 1954), and detailed nucleic acid analyses have revealed a doubling of DNA and RNA on an average cell basis (Scherbaum, 1957*b*). The present study was undertaken in part to determine how nuclear growth is affected by the synchronizing treatment, and the relationship of this process to growth and differentiation of the organism as a whole.

MATERIALS AND METHODS

T. pyriformis GL was grown in 100 ml. of peptone medium in an Erlenmeyer flask as described earlier (Scherbaum & Zeuthen, 1955). At a population density of 50,000–80,000 cells per ml. the heat treatment was started. The treatment lasted 7 hours, during which the cultures were exposed to seven temperature cycles, each consisting of one half hour at 28–29° C. followed by one half hour at 33·9° C. Ten-millilitre samples were removed at regular intervals for study.

The morphological features of the cortex were studied in specimens stained with protargol and with silver nitrate. Protargol preparations were made as follows: cells were fixed in 2 per cent. osmic acid for 2 minutes and passed through ethanol of increasing concentrations. From pure ethanol the cells were transferred to albuminized coverslips. The coverslips with the fixed cells attached were transferred to a 1 per cent. solution of Winthrop–Stern pre-war protargol and allowed to remain in this solution, along with a piece of copper wire, for 24 hours at 22° C. A reducing solution of 1 per cent. hydroquinone in 5 per cent. sulphite was then used. After washing the samples were transferred to a 1 per cent. aqueous gold chloride solution for 5 minutes. The cells were then washed, transferred to aqueous 2 per cent. oxalic acid for 5 minutes, washed again, and transferred to 5 per cent. sodium thiosulphate. Finally, the samples were dehydrated in absolute alcohol, cleared in xylene, and mounted in permount. Hairs were put into the preparations to support the coverslips.

The silver nitrate preparations were made according to the Chatton–Lwoff silver impregnation technique as described by Corliss (1953*a*). In this procedure the cells are embedded in a thin layer of gelatin on a slide prior to impregnation with silver. French gelatin, believed by some workers to be superior to American gelatin for this purpose, was used in the present study. All quantitative data regarding the cortical structures were obtained from such silver nitrate preparations. Kinetosomes were counted in normal and synchronized cultures. For practical reasons, only the number of basal bodies in meridian *n*-2 was determined and this was then used as an index of the total number of somatic kinetosomes present. Meridian *n*-2 is the first meridian to the (animal's) left of the mouth which extends to the apex of the cell. Only those cells oriented in such a way that the entire length of the index meridian was visible were used for counting.

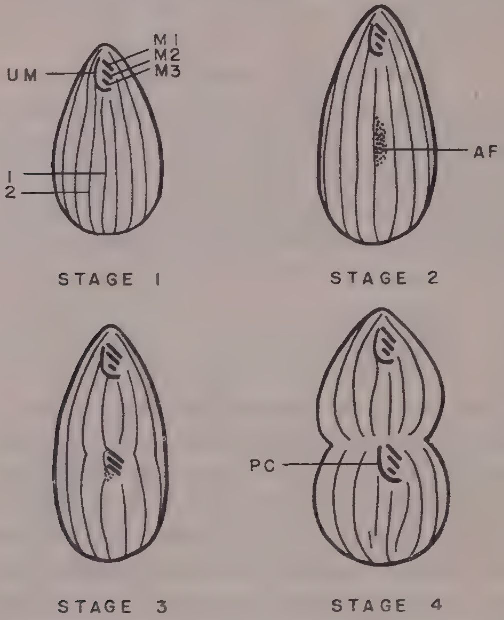
A total of 60 cells was counted in each population considered. In addition samples from various phases of normal and synchronized cultures were analysed for the number of cells possessing anarchic fields of kinetosomes. In each case 150 cells were examined and the number of cells with anarchic fields was expressed on a percentage basis. Finally, the numbers of meridians and contractile vacuole pores were determined in various normal and heat-treated populations. In these cases a sample size of 30 was used for counting.

Macronuclear changes were studied in cells fixed with 1 per cent. osmium tetroxide and stained with the Feulgen nuclear stain. Nuclear diameters were measured in these preparations. For each group under investigation the diameters of 100 nuclei were measured. Because of changes in cell size found in Feulgen preparations the parameters for cell volume and nuclear volume could not be determined on the same cells. Cell volumes were therefore determined on cells fixed in Bouin's fluid. Shrinkage was less than 10 per cent. in this fluid. The major and minor axes of the cells were measured on enlarged photomicrographs. The shape of the cells closely approximated a prolate spheroid, therefore the formula $V = 4/3\pi a^2 b$ was used, where a is the minor axis and b the major axis.

RESULTS AND OBSERVATIONS

Morphology of the normal organisms. The organisms used in the present study agree generally with the detailed descriptions of *T. pyriformis* in the literature (Furgason, 1940; Corliss, 1953*b*). The essential features are the differentiated cortex and the macronucleus (strain GL is devoid of a micronucleus). The differentiated ectoplasmic cortex consists primarily of a cytostome possessing three membranelles and an undulating membrane, eighteen longitudinal rows of cilia, two contractile vacuole pores, and a cytoproct. The classical technique for revealing cortical structure and morphogenesis is silver nitrate impregnation. In the present study the application of protargol, a stain usually associated with studies of flagellated protozoa, has made possible some new observations. The silver nitrate technique has the disadvantage of staining kinetosomes without revealing the major kinetosomal derivatives, i.e. the cilia and ciliary membranes. Protargol was found to stain both of these elements, making possible direct observation of certain morphogenetic activities of the kinetosomes (compare Plate, figs. A and B).

In each meridian of normal cells stained with protargol 3–5 'naked' kinetosomes are usually found, i.e. basal bodies with no cilia attached (Plate, fig. A). These are often smaller than cilium-bearing kinetosomes and often lie close to them. Short cilia have been seen regularly and presumably represent growth stages in the production of cilia by naked kinetosomes. This idea finds support from the fact that cilia of all lengths from short stubs up to the full length of 7 μ have been seen. Such intermediate-length cilia were not found preferentially at any particular body-level, thus suggesting that growth of the somatic ciliature occurs in all regions of the body. The 'fibres' and certain 'granules' seen in silver nitrate preparations are not stained by protargol (Plate, fig. A). The secondary meridians are entirely absent in these preparations. The fibre associated with the primary meridian of silver nitrate preparations has been found by Metz & Westfall (1954) with the electron microscope. This fibre is probably the 'kinetodesma' of Chatton & Lwoff (1935). On the other hand, the only structural feature of *Tetrahymena* found by these workers which corresponds to the secondary meridians of the light microscopist is a series of pellicular rings coursing between the primaries. It therefore appears that silver nitrate stains a variety of structures both on and beneath the pellicle. In contrast to this, protargol stains only the kinetosomes and their cilia. The greater specificity of protargol may present certain advantages for studies in ciliate morphogenesis.



TEXT-FIG. 1. Division stages of *Tetrahymena.* The rows of cilia are indicated by solid lines. The system of numbering for rows of body cilia, or meridians, is indicated in the drawing of stage 1. The stomatogenic meridian is number one and the numbers increase progressively around the animal from left to right. The four mouth membranes are indicated at the anterior region. M1, M2, and M3 are the three membranelles while UM represents the undulating membrane. Stage 2 shows the anarchic field of basal bodies (AF). Stages 3 and 4 indicate formation of the fully developed posterior cytostome (PC).

Developmental stages. It will be useful to divide the process of binary fission into a number of stages. This has been found useful in the study of ciliate morphogenesis by other workers and will here facilitate analysis of the morphological changes which occur in normal and synchronous division. The most marked and easily recognizable changes in fission occur in the cortex. For this reason division stages are defined in terms of cortical events (Text-fig. 1).

Stage 1. From the separation of daughter cells to the beginning of anarchic field formation by meridian number 1, the stomatogenic meridian.

Stage 2. From the beginning of anarchic field formation to the organization of the anarchic field into bands which represent the developing mouth parts.

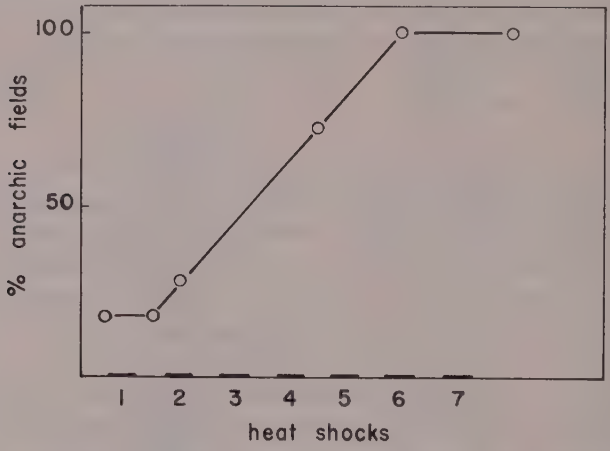
Stage 3. From the organization of the anarchic field into bands to the beginning of cytoplasmic constriction.

Stage 4. From the beginning of cytoplasmic constriction to the separation of daughter cells.

In cells stained with protargol the somatic ciliature at all stages of development showed naked kinetosomes and short cilia. This suggests that somatic kinetosome reproduction and ciliary synthesis occurs at all stages in the cell cycle. Soon after separation the daughter cells elongate considerably. No further morphogenetic events are noted to occur during stage 1. Stage 2 cells stained with protargol indicate that there are no cilia present in the newly formed anarchic fields. The cilia of the undulating membrane and the three membranelles grow out immediately *after* the organization of the anarchic field into bands, i.e. early stage 3. The oral cilia can be seen at all stages of their growth, the young cytostomes with short cilia and older cytostomes with longer cilia. The growing cilia of a given cytostome always appear to be uniform in length, suggesting that their growth is synchronous. The undulating membrane is made of a single row of cilia while the membranelles have two or more rows each. Also during stage 3 the new contractile vacuole pores arise. As protargol stains the macronuclei as well as cortical structures it was possible to correlate nuclear-division stages with the developmental stages defined above. These preparations showed that elongation of the macronucleus begins during late stage 3 of normal cell-division. Stage 4 macronuclei are seen to continue elongation and to pinch in two. Feulgen preparations indicate that by stage 4 the chromatin granules of the macronuclei have increased in size. The coarse-granular condition is typical of the dividing macronucleus. Other events of stage 4 are the formation of the cytopharynx and oral ribs, thus completing the formation of the new cytostome.

Morphogenesis in synchronized organisms. Subsequent to the synchronizing treatment all cells were found to possess large anarchic fields of kinetosomes (Plate, fig. C). This was reported earlier for strain WH-6 (mating type I, variety 1) (Holz, Scherbaum, & Williams, 1957). In the present investigation the frequency of anarchic fields in the population was determined as a function of the synchronizing treatment. The results are presented in Text-fig. 2. Eighteen per cent. of cells in the untreated early maximum stationary phase culture possessed anarchic fields. No increase occurred until the second heat shock. From this time on there was a steady increase until the fifth shock, at which time all cells were found to have anarchic fields. This means that morphologically, 100 per cent.

synchrony is attained by the synchronizing treatment. Protargol preparations of cells upon completion of the treatment showed that no cytostomal cilia were present, indicating the presence of normal stage 2 cytostomes. The cells were found to remain in this condition for a period of 50–55 minutes after completion of the synchronizing treatment. No change in the ectoplasmic cortex could be detected. We refer to this period, therefore, as a period of arrested development. The only change noted to occur during this period was perhaps an increase in the size of the chromatin granules within the macronucleus. Cells examined 1 hour after treatment had resumed development. They possessed normal early stage 3 cytostomes, characterized by the short, stubby, growing cilia arising from the four bands of kinetosomes which are the basal plates of the incipient mouth membranes (Plate, fig. E). A few cells 1 hour after treatment still had anarchic fields present. Since prior to this all cells were in the same stage of development this represents an increase in the morphological variability. Other morphogenetic events occurring after the period of arrested development and prior to cytoplasmic constriction were the production of contractile vacuole pores and the beginning of macronuclear elongation. Cytoplasmic constriction began 1 hour and 20 minutes after treatment. Silver preparations of cells at this time showed a continuing increase in the morphological variability, i.e. while most cells were beginning constriction many others either had stage 3 cytostomes and were not constricted, or else were well along in constriction and had fully formed cytostomes. The silver preparations also showed that the oral ribs and cytopharynx are formed after cytoplasmic constriction is well under way in synchronously dividing cells. Thus, aside from the 50–55-minute period of arrested



TEXT-FIG. 2. Proportion of cells showing anarchic fields of kinetosomes in normal early maximum stationary phase and heat-treated cultures. Each point is based on 150 cells.

development following the synchronizing treatment, the developmental processes in synchronously dividing cells appear to be fundamentally the same as those in normally dividing cells.

Counts were made of the number of meridians and the number of contractile vacuole pores in untreated cells, in cells immediately after the synchronizing treatment, and in cells just prior to the synchronous division. The results are reported in Table 1. It is seen that no change in either meridian number or contractile vacuole pore number results from synchronization. In all cases the modal meridian number was eighteen and the modal contractile vacuole pore number was two.

TABLE 1

Number of meridians and contractile vacuole pores in normal and synchronously dividing organisms

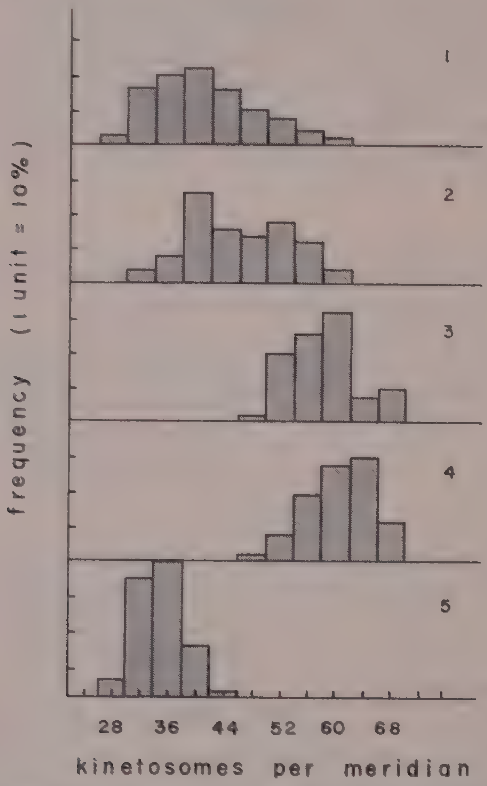
	Untreated cells		*After treatment*		*After induced lag*	
	Meridians	*C.V.P.*	*Meridians*	*C.V.P.*	*Meridians*	*C.V.P.*
Mean	17·68	2·16	17·52	2·2	17·60	2·26
Mode	18	2	18	2	18	2

TABLE 2

Number of kinetosomes in meridian n-2

Sample number	*Mean*	*Range*	*Standard deviation*	*Coefficient of variation*
1 Control, maximum stationary phase	40·14	29–60	9·88	0·246
2 During fourth heat shock .	46·06	33–60	7·14	0·155
3 After seventh heat shock .	57·64	49–68	4·84	0·084
4 Prior to synchronous division	59·86	48–69	4·67	0·078
5 After synchronous division .	34·34	28–43	3·28	0·095

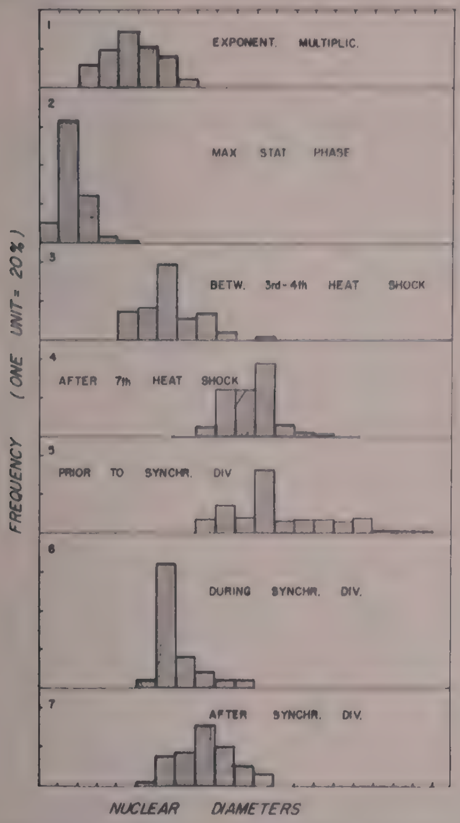
The meridians of synchronized cells were also morphologically similar to the meridians of untreated cells. The distance between the meridians, however, was clearly greater in synchronized cells and is to be expected on the basis of the greater width of synchronized cells reported below. A second meridianal peculiarity of the heat-treated cells is to be noted in the subequatorial regions of meridians 2, 3, and sometimes 4 and 5 (Plate, fig. C). These meridians are arched to the right in this region in association with the 'bulge' or 'notch' previously reported in mating type I, variety 1. Protargol preparations revealed that cells at all periods throughout treatment and the subsequent synchronous cell-division possessed naked kinetosomes and short cilia. Although no quantitative data were obtained it can be stated that no obvious alteration of the frequencies of these two elements was noted.



TEXT-FIG. 3. Number of kinetosomes in meridian *n*-2 in normal and synchronized cultures (excluding cells in the process of cytoplasmic constriction). The following populations are represented: early maximum stationary phase cells (1), during the fourth heat shock (2), after the last heat shock (3), prior to synchronous division (4), and after the synchronous division (5). The synchronizing treatment increases the mean number of kinetosomes while reducing the coefficient of variation. Each histogram is based on 60 cells.

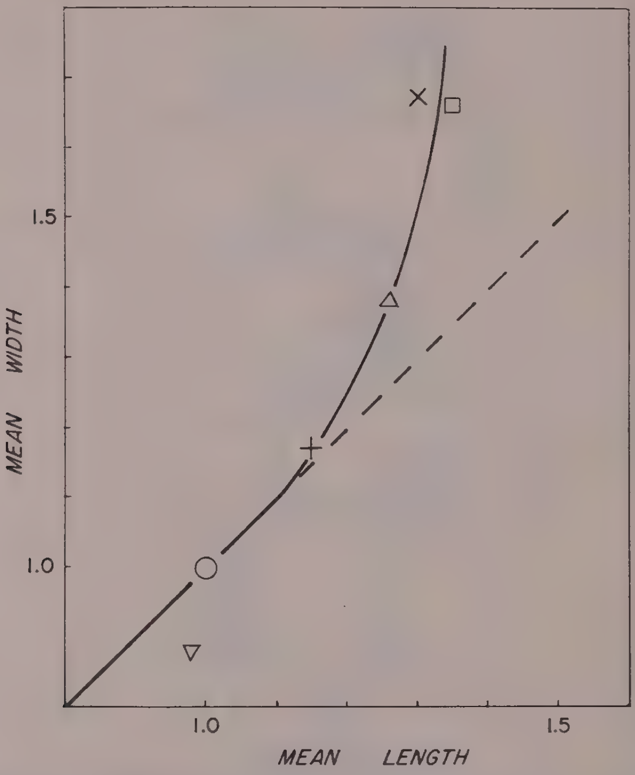
The number of kinetosomes in meridian *n*-2 was determined for individuals in populations at various times during and after treatment. These data are presented in Text-fig. 3. It should be emphasized that it was not possible to count the number of kinetosomes in the meridians of cells in the process of cytoplasmic constriction. The bias thus introduced will be considered in the discussion. It is seen from Table 2 that while the mean number of kinetosomes in meridian *n*-2 gradually increased from 40 to 57 during the synchronizing treatment, the variance progressively *decreased*. The coefficient of variation decreased from

0·246 in the untreated cells to 0·084 after the seventh heat shock. There is very little change in the mean and variance during the long induced lag between the end of treatment and the resumption of development. The synchronous fission which follows reduces the mean to 34 kinetosomes. The standard deviation is also reduced but the coefficient of variation is slightly increased. An interpretation of these data will be presented in the discussion.



TEXT-FIG. 4. Nuclear diameters at different stages of normal and synchronized cultures. Each histogram is based on the measurement of 100 nuclei.

Growth in heat-treated organisms. Nuclear size was determined in logarithmic phase cells, stationary phase cells, and at various times during and after the synchronizing treatment. These data are presented in arbitrary units representing nuclear diameters in Text-fig. 4. It is seen from this histogram that stationary phase cells had nuclei considerably smaller than cells in logarithmic phase of growth. A similar observation was reported previously by Summers, Bernstein, & James (1957). After three heat shocks in the synchronizing treatment the nuclear size was increased. This increase continued until the nuclei after treatment were approximately three times as large as normal logarithmic phase cells (Table 3). Interestingly, some of the already greatly oversized nuclei continued to increase in size after treatment and prior to the synchronous cytoplasmic constriction. It is not known whether this growth takes place during the period of arrested development, subsequent to this, or during both periods since the sample was taken immediately prior to cytoplasmic constriction. During the simultaneous division the nuclear volumes become remarkably uniform. Sixty-four per cent. of the measured nuclei fall into one size class. The mean volume is also decreased during the synchronous division more than the expected one-half. This implies a change in density of the nuclear material or a loss of nuclear



TEXT-FIG. 5. Mean cell width and mean cell length as a function of the temperature treatment. The mean width and mean length of the average normal cell (marked ○ in the figure) are arbitrarily set equal to 1 for comparison with the mean cell length and width at various growth phases. Designation of the other symbols: + between third and fourth heat shock, × after seventh heat shock, □ prior to division, △ after synchronous division, ▽ maximum stationary phase. The broken line indicates a constant width/length ratio. Each point is the average of measurement of 300 cells.

material into the cytoplasm. The Feulgen preparations indicate such a loss of macronuclear chromatin to the cytoplasm during the synchronous division. Quantitative consideration of this extranuclear chromatin will appear in a separate study. After division the relative size of the average macronucleus readjusts itself to a value close to one-half that found just prior to the synchronous division.

TABLE 3

Nuclear volumes in normal and synchronized cultures

(The mean volume is given in arbitrary units at the left. In the right column under 'Mean volume' the volume of untreated cells is taken as 1 and the other populations compared to this)

Sample	*Mean volume*		*Range*	*Standard deviation*	*Coefficient of variation*
Control, exponential multiplication .	265	1·00	149–458	80	0·30
Between third and fourth heat shock .	397	1·50	246–898	118	0·30
After seventh shock	816	3·08	553–1,554	166	0·20
Prior to synchronous division . .	1,063	4·01	553–3,328	460	0·43
During synchronous division . .	430	1·62	308–769	101	0·24
After synchronous division . . .	571	2·16	380–898	142	0·25
Maximum stationary phase . . .	122	0·46	82–246	26	0·22

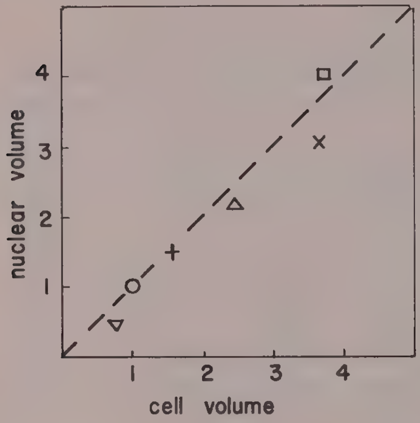
TABLE 4

Cell volumes in normal and synchronized cultures

(To facilitate comparison the values for length, width, and volume of the normal average cell is also taken arbitrarily as 1)

Sample	*Mean length*		*Mean width*		*Mean volume*	
	μ		μ		μ^3	
1 Control, exponential multiplication	51·1	1·00	30·8	1·00	25,380	1·00
2 Between third and fourth heat shock	58·8	1·15	36·0	1·17	39,900	1·57
3 After the seventh shock . .	66·4	1·30	51·6	1·67	92,590	3·65
4 Prior to synchronous division .	69·0	1·35	51·1	1·66	94,370	3·72
6 After synchronous division . .	64·3	1·26	42·6	1·38	61,360	2·42
7 Maximum stationary phase . .	50·3	0·98	27·1	0·88	19,340	0·76

Cell size was determined in normal and heat-treated cultures and the nuclear size data related to this. Stationary phase cells show a decreased width–length ratio while heat-treated cells show an increased width–length ratio when compared with cells in the logarithmic phase of growth (Text-fig. 5). The data on cell volumes is reported in Table 4. While the nuclei of stationary phase cells are reduced to approximately one-half the volume of logarithmic phase cells, the total cell volume is reduced by less than this amount. The average stationary phase nucleus, therefore, is smaller in relation to the total cell volume than the average logarithmic phase nucleus (Text-fig. 6). In the course of the heat treatment the mean cell volume increased more on a percentage basis than the mean macronuclear volume. During the time subsequent to the treatment and prior to the synchronous cytoplasmic constriction the cell volume increased only slightly, while the macronuclear volume increased 30 per cent. These shifts in nucleo-cytoplasmic relations can be visualized from Text-fig. 6. After the synchronous cell-division the nuclear size was found to be reduced in relation to the cell size. The nuclear volume was reduced by approximately half by the synchronous division but the cell volume was reduced by less than this proportion.



TEXT-FIG. 6. Changes of nuclear versus cell volume at different growth stages in arbitrary units. Normal cell volume and nuclear volume (marked ○ in the figure) are both taken as one. Designation of the other symbols: + between the third and fourth heat shock, × after the seventh heat shock, □ prior to division, △ after the synchronous division, ▽ maximum stationary phase of growth in untreated cells. The broken line indicates a constant nucleo-cytoplasmic ratio. Each point is based on the measurement of 300 cells and 100 nuclei.

DISCUSSION

Analysis of cortical differentiation in cultures of *T. pyriformis* GL subjected to the synchronizing treatment has indicated that the cells are temporarily arrested in their development, but are otherwise structurally normal. The normal sequence of morphogenetic events associated with binary fission has been blocked at a characteristic point in these cells, i.e. at late stage 2. The specific developmental processes which occur in treated cells prior to the block and subsequent to the continuation of development in the synchronous division are fundamentally the same as for normal binary fission. Treated cells have the same number of meridians, the same number of contractile vacuole pores, and the same cortical anatomy as cells from normal cultures that are in the same stage of the cell cycle. The number of kinetosomes should be discussed in this connexion.

The larger number of kinetosomes found in cells subjected to the synchronizing treatment does not appear to be the result of an abnormal increase. The range for the number of kinetosomes in meridian *n*-2 in untreated cells was from

29 to 60. It was observed that the low end of the distribution represents young cells and the high end represents cells just prior to cytoplasmic constriction (kinetosome counts could not be carried out on cells undergoing constriction). This means that the number of kinetosomes in meridian *n*-2 in late stage 3 untreated cells is in the neighbourhood of 60. The mean number of kinetosomes in meridian *n*-2 for cells in late stage 3 of the synchronous division was 59·86. This suggests that the number of kinetosomes in cells after the synchronizing treatment is the same as the number of kinetosomes in untreated cells that are at a *comparable stage in the cell cycle*. The greater mean number of kinetosomes in meridian *n*-2 in synchronized cells is therefore a result of the fact that all cells are brought into synchrony at late stage 2, a period in the cell cycle characterized by a large number of somatic kinetosomes. A second feature of the frequency distribution of the number of kinetosomes in meridian *n*-2 of heat-treated cells is the reduced coefficient of variation. Since the number of kinetosomes increase as the cells approach the division stage, much of the variability in kinetosome numbers in a normally growing population will be due to the distribution of cells at various points in the cell reproductive cycle. A reduction in the coefficient of variability is thus to be expected when any degree of synchrony is induced in the population.

The mean number of kinetosomes in meridian *n*-2 was 57·64 after the synchronizing treatment. The cells resumed development in synchrony and were found to possess a mean number of 59·86 kinetosomes in meridian *n*-2 just prior to cytoplasmic constriction, i.e. at late stage 3. As it was not possible to count kinetosomes in cells undergoing cytoplasmic constriction there is no direct evidence that there is a kinetosome increase during this stage. However, the mean number of 34·34 kinetosomes found in meridian *n*-2 in cells just after the synchronous division suggests that during stage 4 there may have been an increase from 59·86 to twice the number found in the daughter cells, i.e. 68–68. The data from synchronously dividing *T. pyriformis*, therefore, suggest that the growth of the somatic ciliature may be most rapid during cytoplasmic constriction and after, with a reduction in the rate of increase occurring prior to constriction. Interestingly, this type of increase with respect to the cell cycle of *Tetrahymena* has been reported for respiration by Zeuthen (1953).

The period of arrested development was found to occupy about 50–55 minutes. With the exception of an increase in the size of the chromatin granules of the macronucleus, no changes were noted to occur during this period. Previous investigations have led to the general conclusion that the blockade to cell-division in heat-treated cells may be due to a reversible denaturation of a single enzyme system (Scherbaum, 1957*a*). From the present study it would appear that the enzyme system in question interferes with morphogenesis in a non-specific way, affecting the developmental sequence as a whole but not selectively inhibiting any constituent process. This does not mean, however, that a single developmental event cannot be the point of action of the synchronizing treatment. This could actually be the case if all events normally occurring after stage 2 were dependent upon the integrity of this step. Biochemical investigations of arrested development in *Tetrahymena* might be rewarding.

An interesting feature of synchronized cultures of *Tetrahymena* is the dissociation of nuclear growth, cell growth, and morphogenesis. 'Growth' as used here refers to increases in volume. During the synchronizing treatment the cells apparently proceed to developmental stage 2 and stop, while nuclear and cell volumes continue to increase. During the period subsequent to the synchronizing treatment and prior to the onset of cytoplasmic constriction there is an appreciable increase in nuclear volume with no concurrent increase in cell volume. It is not known whether this nuclear growth takes place during the period of arrested development, after this period, or at both times, since the measured sample was taken just prior to cytoplasmic constriction. However, a 27 per cent. increase in DNA has been reported to occur during a *one-hour* interval subsequent to the synchronizing treatment (Scherbaum, 1957*b*), suggesting that most of the nuclear volume increase and the DNA synthesis may occur during arrested development. If this is correct we may conclude that the extensive morphogenesis which occurs in all cells synchronously after the period of arrested development takes place in the absence of any appreciable increase in nuclear and cell volume.

SUMMARY

1. A study of differentiation in the ectoplasmic cortex of *T. pyriformis* GL subjected to the standard temperature cycling for the induction of synchronous binary fission has demonstrated an arrest in development which occurs in all cells at a characteristic point in the cell cycle. After treatment all cells were found to possess anarchic fields of kinetosomes in the stomatogenic region, indicating 100 per cent. synchrony. The cells remain in this condition for a period of 50–55 minutes after the last heat shock. During this time no changes other than in increase in the size of the macronuclear chromatin granules could be detected. At the end of the period of arrested development morphogenesis resumes and binary fission continues in synchrony. The degree of synchrony is somewhat reduced as development proceeds, resulting in about 85 per cent. synchrony at the time of cytoplasmic constriction.

2. In order to facilitate analysis of morphogenesis in synchronously dividing *Tetrahymena*, a series of four developmental stages has been defined. Based on work which has confirmed available descriptions of stomatogenesis and macronuclear fission, the sequence includes new data on development of the somatic ciliature. All observations, including quantitative consideration of the somatic kinetosomes, ciliary meridians, and contractile vacuole pores has indicated that, aside from synchrony and the period of arrested development, the morphogenetic events in synchronized organisms are fundamentally no different from these processes in untreated cultures.

3. Growth of the organism, growth of the macronucleus, and morphogenesis are to some extent dissociated in synchronized cultures of *Tetrahymena*. During the heat treatment morphogenesis is blocked while cell and macronuclear growth continues. After treatment and prior to the synchronous cytoplasmic constriction there is a significant increase in mean nuclear volume with no significant increase in total cell volume. After resumption of development in synchronized cultures morphogenesis proceeds unaccompanied by any increase in cellular volume. In addition, it is believed that the nuclear increase occurs during the period of arrested development and not during the subsequent period of development.

4. The use of protargol staining promises to be of value in studies of ciliate morphogenesis. Of those structures in and on the ciliate cortex, protargol appears to be highly specific for kinetosomes. In addition to this the cilia and ciliary membranes are revealed by this technique.

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EXPLANATION OF PLATE

FIG. A. Photomicrograph of a normal individual stained with protargol, showing cytostome (C) and ciliary rows. Both the cilia and their basal bodies (kinetosomes) are revealed by this technique. Note kinetosomes without cilia found in various regions of the body (NK). These may represent newly formed kinetosomes prior to ciliary synthesis.

FIG. B. Photomicrograph of a normal individual stained according to the Chatton–Lwoff silver impregnation technique. Kinetosomes and fibrils are revealed but not the cilia or ciliary membranes. Meridian *n*-2 was used for kinetosome counts and is designated in this photomicrograph. This particular cell shows 32 kinetosomes in meridian *n*-2, indicating that the organism is in the early part of the cell cycle.

FIG. C. Photomicrograph of a cell impregnated with silver immediately after the synchronizing treatment. Note the anarchic field of kinetosomes (AF) present in the equatorial region and the large numbers of kinetosomes in the meridians. This is the condition in all cells subsequent to treatment, i.e. 100 per cent. synchrony has been obtained. For purposes of orientation it should be noted that the ventral surface has been photographed from the dorsal side of this organism. Thus, in this case, the animal's right is on the viewer's right.

FIG. D. Photomicrograph of a silver-impregnated cell 1 hour and 10 minutes after the synchronization treatment. The kinetosomes of the anarchic field have organized into the pattern characteristic of the fully developed cytostome, indicating that development has been resumed. The meridianal pattern in the region of the developing cytostome foreshadows the beginning of constriction which began 10 minutes later in the population from which this individual was taken. Nearly all cells in the population were at this stage of development, although synchrony was no longer 100 per cent.

FIG. E. Photomicrograph of a protargol-stained cell at approximately the same stage in the synchronous division as the above. At this time the growing cilia of the developing undulating membrane (DC) and membranelles are still shorter than the neighbouring somatic cilia.

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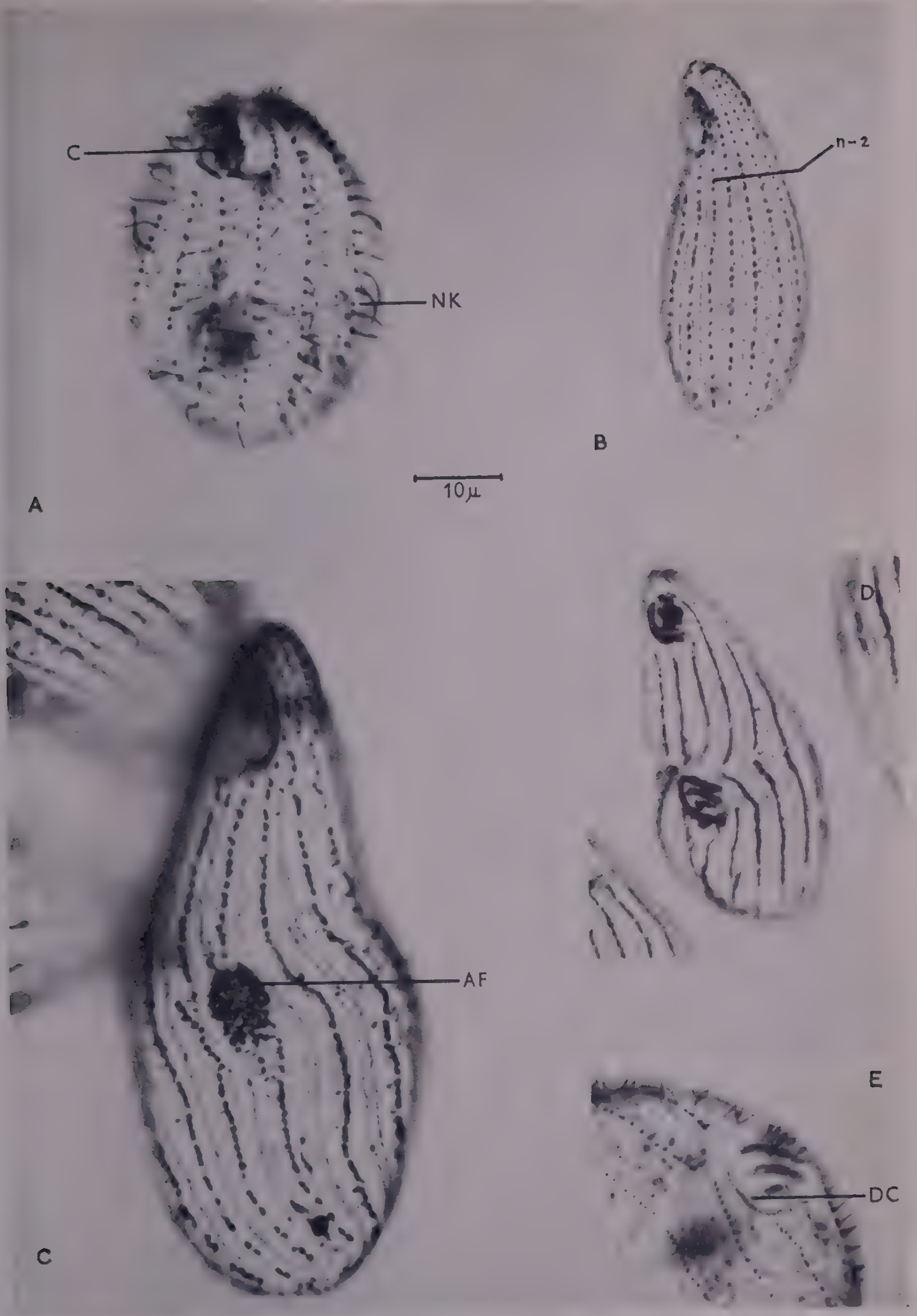

N. E. WILLIAMS *and* O. H. SCHERBAUM

A Study of Environmental Influences on the Size of the Pronephros and Related Structures in *Triturus helveticus*

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INTRODUCTION

IT is well known that when one pronephros of a urodele is removed, or if its duct is blocked, the other undergoes compensatory hypertrophy. Previous papers (Fox, 1956, 1957) have provided a quantitative analysis of this process in *Triturus cristatus* and *T. helveticus*. The present study is concerned with the intact pronephric system of embryos reared in various environments, in the hope that additional light might be thrown on the variables affecting pronephric growth. The factors studied were the osmotic concentration, the presence or absence of calcium ions, and the nitrogen concentration in the surrounding medium. Consideration was also given to the effects of decapsulation. One result of particular interest was the fact that pronephroi were significantly larger when young urodele larvae developed in a fluid medium of reduced ionic concentration.

MATERIAL AND METHODS

Embryos of *T. helveticus* investigated were of two different years and quite unrelated. Those of collection A, obtained from Dr. Helen Spurway, were spawned in the spring of 1954 from several pairs maintained at University College. Embryos of collection B were spawned in the spring of 1957 from eight breeding pairs obtained from a commercial dealer. All embryos were immersed in the chosen media when about 2·0 mm. long, i.e. equivalent to Harrison stage 24–26 for *Ambystoma punctatum* (Hamburger, 1950). Collection A comprised experimental groups 1–4 and collection B groups 5–9. Embryos of groups 2 and 6 remained in their capsules; those of all other groups were decapsulated on immersion. Development of groups 1 and 2 took place in Holtfreter solution diluted ten times (Holtfreter 1/10); group 3 developed in Holtfreter solution; group 4 in distilled water. Development of groups 5 and 6 took place in Holt-

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freter 1/10; group 7 in Holtfreter solution; group 8 in calcium-free Holtfreter 1/10, the calcium chloride being replaced by an osmotically equivalent quantity of sodium chloride; and group 9 developed in Holtfreter 1/10 containing 0·5 per cent. urea. All specimens were kept in glass crystallizing dishes, four to each dish, in about 75 ml. of liquid. One inch of air space lay between the solution surface and the glass dish cover. Solutions were changed every 24 hours and the temperature varied between 18° and 20° C. *Cyclops* and *Daphnia* were added to the medium after 12 days, when feeding became possible. After 15 days' treatment seven larvae, each about 8·0 mm. long, were chosen at random from each group (one extra specimen from each group was sectioned horizontally to measure pronephric nuclear length), and killed in Smith's fixative. Histological and quantitative methods were those previously described (Fox, 1956, 1957). The level of statistical significance used throughout was $P < 0{\cdot}05$. There were no obvious differences in rate of development, judged from external appearance, between the specimens of each year.

Holtfreter solution has the following composition: NaCl 0·35 g., $CaCl_2$ 0·01 g., $NaHCO_3$ 0·02 g., KCl 0·005 g., per 100 ml. distilled water. The depression of freezing-point, $\Delta = 0{\cdot}22°$ C. (Holtfreter, 1931), corresponds to an osmotic pressure of about 2·7 atmospheres at 0° C. A 0·5 per cent. solution of urea in distilled water has an osmotic pressure of 1·87 atmospheres at 0° C. (see Cushny, 1926). Cell membranes are, however, freely permeable to urea, and this substance rapidly ceases to exert any osmotic effects.

RESULTS

Collection A

Effect of decapsulation (groups 1 and 2). No significant differences were found between the pronephric systems of the two groups reared in Holtfreter 1/10, one decapsulated (group 1), the other not (group 2), except in total lumen volume and internal surface area of the tubules (Table 1). Means of these measurements in the decapsulated embryos were significantly smaller by about one-fifth. The general histological pattern is otherwise similar.

Comparison of decapsulated embryos reared in Holtfreter 1/10 (group 1) and Holtfreter solution (group 3). No significant differences were found between the two groups (Table 1).

Comparison of decapsulated embryos reared in Holtfreter solution (group 3) and in distilled water (group 4). The mean of every measurement made, except that for nuclear length, is significantly and considerably larger in the distilled water group than in group 3; for example the total volumes of cells, lumina, and overall volume, are increased by 90 per cent., 72 per cent., and 86 per cent. respectively (Table 1). These quantitative differences are reflected in the histological appearance of the pronephric systems. Both pronephric tubules and duct are wider in group 4, and have thicker walls.

TABLE 1

Various measurements of the nine groups of T. helveticus *from collections A and B*

Each group included 14 pronephroi and glomi from 7 larvae. Larvae were approximately 8·0 mm. long.

	Group 1. Decapsulated. Holtfreter × 10⁻¹	*Group 2. Capsulated. Holtfreter* × 10⁻¹	*Group 3. Decapsulated. Holtfreter*	*Group 4. Decapsulated. Distilled water*	*Group 5. Decapsulated. Holtfreter* × 10⁻¹	*Group 6. Capsulated. Holtfreter* × 10⁻¹	*Group 7. Decapsulated. Holtfreter*	*Group 8. Decapsulated. Ca-free*	*Group 9. Decapsulated. 0·5 per cent. urea*
Mean nose-to-cloacal length (mm.)	4·36 ± 0·042	4·35 ± 0·063	4·24 ± 0·056	4·26 ± 0·033	4·51 ± 0·054	4·41 ± 0·083	4·48 ± 0·063	4·36 ± 0·051	4·37 ± 0·042
Mean antero-posterior length of pronephros (mm.)	0·301 ± 0·004	0·311 ± 0·004	0·292 ± 0·011	0·374 ± 0·009	0·376 ± 0·007	0·416 ± 0·014	0·392 ± 0·014	0·377 ± 0·010	0·403 ± 0·008
Mean nuclear population of pronephros	590 ± 17	574 ± 13	557 ± 32	742 ± 27	636 ± 18	597 ± 19	634 ± 20	590 ± 22	601 ± 16
Mean total volume of cells of pronephros ($mm.^3 \times 10^3$)	2·1 ± 0·09	2·2 ± 0·09	1·9 ± 0·12	3·6 ± 0·10	4·0 ± 0·29	4·1 ± 0·23	3·1 ± 0·18	3·9 ± 0·14	4·3 ± 0·13
Mean total volume of lumina of tubules ($mm.^3 \times 10^3$)	0·8 ± 0·06	1·1 ± 0·05	0·9 ± 0·10	1·55 ± 0·09	2·1 ± 0·21	4·0 ± 1·07	3·9 ± 1·02	1·6 ± 0·09	1·5 ± 0·12
Mean overall volume of pronephros ($mm.^3 \times 10^3$)	3·0 ± 0·12	3·2 ± 0·11	2·8 ± 0·21	5·2 ± 0·17	6·1 ± 0·48	8·1 ± 1·26	6·9 ± 1·18	5·5 ± 0·20	5·8 ± 0·22
Mean area of internal surface of tubules ($mm.^2$)	0·1423 ± 0·0058	0·1714 ± 0·0067	0·1373 ± 0·0094	0·2233 ± 0·0082	0·2576 ± 0·0132	0·2978 ± 0·0369	0·2661 ± 0·0275	0·2247 ± 0·0086	0·2306 ± 0·0091
Mean volume of individual cell of pronephros (μ^3)	3674 ± 175	3788 ± 161	3377 ± 91	4871 ± 66	6305 ± 293	6860 ± 329	4784 ± 161	6573 ± 173	7122 ± 153
Mean antero-posterior length of 240 pronephric nuclei in horizontal section (μ)	12·255 ± 0·179	12·046 ± 0·180	12·065 ± 0·186	12·046 ± 0·175	12·084 ± 0·215	12·122 ± 0·199	12·027 ± 0·194	12·008 ± 0·192	11·970 ± 0·181
Mean diameter of nephrostome base (including walls) of anterior and posterior nephrostomes together (mm.) (total of 28 nephrostomes measured)	0·0379 ± 0·0012	0·0396 ± 0·0013	0·0393 ± 0·0011	0·0464 ± 0·0018	0·0475 ± 0·0014	0·0454 ± 0·0014	0·0443 ± 0·0015	0·0468 ± 0·0016	0·0439 ± 0·0017
Mean diameter of anterior nephrostome base (mm.)	0·0414 ± 0·0014	0·0436 ± 0·0013	0·0429 ± 0·0013	0·0521 ± 0·0021	0·0500 ± 0·0018	0·0479 ± 0·0021	0·0500 ± 0·0015	0·0514 ± 0·0014	0·0486 ± 0·0023
Mean diameter of posterior nephrostome base (mm.)	0·0342 ± 0·0014	0·0357 ± 0·0017	0·0357 ± 0·0014	0·0407 ± 0·0020	0·0450 ± 0·0020	0·0428 ± 0·0016	0·0386 ± 0·0014	0·0421 ± 0·0024	0·0393 ± 0·0019
Mean antero-posterior length of glomus (mm.)	0·166 ± 0·0069	0·179 ± 0·0071	0·169 ± 0·0063	0·212 ± 0·0046	0·234 ± 0·0069	0·241 ± 0·0054	0·237 ± 0·0099	0·248 ± 0·0067	0·234 ± 0·0057
Mean total volume of glomus ($mm.^3 \times 10^3$)	0·43 ± 0·020	0·43 ± 0·016	0·38 ± 0·019	0·59 ± 0·024	0·59 ± 0·026	0·66 ± 0·035	0·52 ± 0·020	0·60 ± 0·040	0·60 ± 0·038
Mean total external surface area of glomus ($mm.^2$)	0·0316 ± 0·0014	0·0329 ± 0·0011	0·0305 ± 0·0011	0·0422 ± 0·0012	0·0450 ± 0·0015	0·0488 ± 0·0016	0·0443 ± 0·0017	0·0471 ± 0·0020	0·0472 ± 0·0019

Collection B

Effect of decapsulation (groups 5 and 6). No significant differences were found between the pronephric systems of decapsulated (group 5) and capsulated (group 6) specimens reared in Holtfreter 1/10, except for the mean antero-posterior pronephros length of capsulated specimens, which was significantly greater (by about 11 per cent.) than that of decapsulated specimens (Table 1).

Comparison of decapsulated embryos reared in Holtfreter 1/10 (group 5) and in Holtfreter solution (group 7). In group 7 the means of the total volume of pronephric cells (22·5 per cent.), the calculated individual cell volume (24 per cent), the diameter of the posterior nephrostomial tubule base (14 per cent.), and the glomus volume (12 per cent.) were significantly lower by the stated percentages than those in the Holtfreter 1/10 group (Table 1). These results may be compared with those obtained in the same experiment in collection A where, though no significant differences were found, total pronephric cell volume, calculated individual cell volume, and glomus volume were likewise reduced.

Comparison of decapsulated embryos reared in Holtfreter 1/10 with (group 5) and without (group 8) calcium chloride. There are no significant differences between the measurements of the various pronephric components, except in the means of tubule lumen volumes and internal surface area, both of which were lower in group 8 (by about 24 per cent. and 13 per cent. respectively) than the corresponding measurements in group 5 (Table 1).

Comparison of decapsulated embryos reared in Holtfreter 1/10 without (group 5) and with (group 9) 0·5 per cent. urea. No significant differences between measurements of the two groups were found, except that in the group reared in the presence of urea the means of the antero-posterior pronephric lengths and calculated individual cell volumes were slightly, but significantly, greater, by 7 per cent. and 13 per cent. respectively, and the lumen volume smaller by 29 per cent. (Table 1).

Comparison of embryos of the two different collections decapsulated and reared in Holtfreter 1/10 (groups 1 and 5). Specimens of both groups are generally similar in size and appearance after 15 days' treatment, but the mean nose-to-cloacal length measurement in group 5 is significantly greater (by 3·4 per cent.) than in group 1. All specimens possess trifid forelimbs, three pairs of feathery external gill filaments, and an open mouth. The mean measurement of every pronephric component, except for nuclear length and total nuclear population, is significantly greater in group 5 than in group 1. These differences seem bigger than would be expected, even if there was some slight age difference (see Table 1).

DISCUSSION

Pronephroi of decapsulated embryos of collection A have a significantly smaller lumen volume and internal surface area than have capsulated embryos; similar, but not significant, reductions were found in collection B, but here there is a significantly smaller pronephric length. The greater tubule lumen volume of capsulated embryos may be a consequence of the embryos' U-shape before hatching. The lumina of the pronephric ducts are therefore narrowed, and this may lead to a higher intra-tubular pressure than in decapsulated 'straight' larvae, and cause tubule inflation. If this hypothesis were correct an increase in mitotic rate and hence in nuclear population would be expected (Fox, 1957), but this did not occur.

Pronephroi of collection A were generally (but not significantly) smaller in specimens reared in full-strength Holtfreter solution than in those reared in tenfold diluted medium. In collection B, embryos in full-strength Holtfreter had a significantly smaller mean total cell volume, calculated individual cell volume, and glomus volume. When embryos are reared in full-strength Holtfreter, which is hypertonic to the body fluids of the embryo (Hamburger, 1950, p. 26) as well as to the intra-capsular fluid of *Ambystoma punctatum* (Richards, 1940), water will diffuse through the skin out into the surrounding medium (Jenkinson, 1906); it would therefore be withdrawn from the pronephric and glomus tissues, thus reducing their volumes. Nevertheless, ions will presumably migrate through the skin into the embryo and so raise the osmotic pressure of the body fluids, for ions diffuse through the skin into the adult frog (Przylecki, 1922; Adolph, 1927*b*, 1933; Krogh, 1937), and larvae readily absorb sodium and chlorine ions (Krogh, Schmidt-Nielsen, & Zeuthen, 1938) at the time of appearance of the external gills (Krogh, 1939). In contrast, however, Jenkinson (1906) considered that frog larvae aborb little salt. Ionic absorption may well account for the failure to obtain significant reductions of pronephros and glomus volumes in collection A.

The effect of rearing embryos in distilled water might be expected, *a priori*, to result in pronephric and glomus enlargement, and this was found to be the case. In distilled water, osmotic effects—which influence volume changes in amphibian larvae (Adolph, 1927*a*; Krogh, Schmidt-Nielsen, & Zeuthen, 1938)—operate, and more water will enter an embryo reared in this medium than one reared in full-strength Holtfreter solution. Leakage of ions out of the embryo (Krogh, 1939) into the distilled water presumably is small, as such embryos appear normal in behaviour and appearance. Surplus water would dilute the embryo body fluid, were it not for the increased activity of the pronephros, and increased water absorption by the pronephros may be a factor in tissue volume increase. Surplus coelomic fluid directed through the pronephros would increase intra-tubular tension, and tension can stimulate mitosis (Abercrombie, 1957). Raised intra-tubular tension will stretch the tubules, increase lumen volume and internal surface area, and provide a larger surface for absorption. Unilateral pronephrectomy, which elicits compensatory hypertrophy by a remaining pronephros, and the subjection of paired pronephroi to an increased functional load, both evoke a similar response.

The presence or absence of calcium ions in the surrounding medium did not appear to play any significant role in influencing pronephric size—except for the significant lowering of lumen volume and internal surface area in embryos reared in calcium-free Holtfreter 1/10. Calcium deficiency in the surrounding medium does not seem to increase skin permeability for water in urodele embryos.

About 90 per cent. of the nitrogen excreted by amphibian embryos is in the form of ammonia (Cambar, 1947). It is particularly toxic to developing amphibia (Jenkinson, 1906) and the ammonia excreted is extremely dilute. Accordingly urea, which is much less toxic, was used to study the effect of a high nitrogenous load on the pronephros. Embryos reared in 2 per cent. urea in distilled water curled up and were malformed, but when reared for 15 days in 0·5 per cent. urea in Holtfreter 1/10 they are apparently normal, with pronephroi of slight, but significant, superiority in mean length and calculated cell volume but of lower lumen volume compared with the controls. Urea is probably an unimportant factor in normal amphibian pronephric development because of the small quantity excreted.

Any small age differences, even if they existed, could hardly account for the disproportion in size between the pronephric systems of the two different collections of *Triturus* (compare groups 1 and 5). The two groups thus show quantitative intra-specific differences between their pronephric systems; larvae of group 1 (collection A) have the smaller variances in the measurements of their pronephric components, probably because they are genetically the more homogeneous group.

SUMMARY

1. *Triturus helveticus* embryos were reared from a stage equivalent to Harrison stage 24–26 for *Ambystoma punctatum*, in a variety of media for 15 days, and the pronephric systems were quantitatively analysed.

2. The lumen volumes and internal surface areas of the tubules of decapsulated embryos were smaller than those of capsulated embryos.

3. The pronephric tissue volumes and glomus volumes of decapsulated embryos reared in full-strength Holtfreter solution were smaller than when they were reared in one-tenth-strength Holtfreter solution.

4. All measurements of pronephric components (except nuclear length) were significantly and considerably greater in decapsulated specimens reared in distilled water than in decapsulated embryos reared in full-strength Holtfreter solution.

5. Pronephroi of decapsulated embryos reared in calcium-free one-tenth-strength Holtfreter solution had a significantly smaller lumen volume and internal surface area of the tubules than those of controls.

6. Pronephroi of decapsulated embryos reared in 0·5 per cent. urea in one-tenth-strength Holtfreter solution had slight, but significant, superiority in length and cell volume, but a smaller lumen volume, than those of controls.

7. Comparison of the pronephric measurements of two unrelated collections of *T. helveticus* showed significant intra-specific differences.

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Appearance of Antigens during Development of the Lens

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WITH TWO PLATES

INTRODUCTION

THOSE cells of the head ectoderm of chick embryos which are to give rise to the lens show the first signs of differentiation at the 13–16-somite stage: the nuclei become cylindrical and move gradually toward the base of the cells, while the vacuoles present in the cytoplasm decrease in size and number. During the 16–20-somite stage the changes become clear-cut as the cells transform from a cuboidal to a high cylindrical shape and the nuclei elongate and move perpendicularly to the retinal surface (*placode formation*; Plate 1, fig. 1*b*; McKeehan, 1951; Van Doorenmaalen, 1958; Langman *et al.*, 1957).

During the 20–30-somite stage the lens placode invaginates and gradually forms a lens vesicle which remains attached to the ectoderm until the 30–31-somite stage, but separates from it at the 32–33-somite stage (*lens vesicle formation*; Plate 1, figs. 1*c*, 2*b*, 2*c*). At the cellular level it was noted that acidophilic fibres appear in the apical cytoplasm at the 23–24-somite stage. During subsequent development (26–30 somites) these fibres become longer, while the fine basophilic granules appear at the retina-facing base of the cells. A slight elongation of the cells noted at the same time continues to form the first so-called nuclear lens fibres (32–37-somite stage; Plate 2, fig. 3*b*). Later, at the 44-somite stage, the lumen of the lens vesicle becomes crescent-shaped (Plate 2, fig. 4*b*).

At the end of the 4th day of incubation (44 somites) the cells in the marginal zone of the lens show high activity such as enlargement of the nuclei, cell division, appearance of basophilic granules in the base of the cells, and acidophilic fibres in the apical cytoplasm. The cells change from cuboidal to high cylindrical, turn 130 degrees, and elongate into new lens fibres. These fibres join the lens nucleus and form the marginal lens fibres. The formation of these fibres continues until the adult stage.

After Uhlenhuth (1903) discovered the strong antibody-forming properties of lens proteins many workers (Burke *et al.*, 1944; Ten Cate & Van Doorenmaalen,

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1950; Woerdeman, 1953, 1955; Langman *et al.*, 1957, 1959; Van Doorenmaalen, 1958) have attempted to obtain information on the chemical development of the lens by means of antigen-antibody techniques. They hoped to detect at which stage of development lens antigens arose and what the relation between morphological and chemical differentiation was. Using micro-precipitin methods, Ten Cate and Van Doorenmaalen (1950) were able to demonstrate the presence of 'adult' lens antigens in lens vesicle extract from 30-somite chick embryos (cf. Plate 1, fig. 2*b*). Woerdeman (1953, 1955), applying the same technique, was able to demonstrate the presence of adult lens antigens in a mixture of equal parts of ectoderm and eye vesicles extract obtained from young axolotl neurulae. The precipitin technique of Boyd (1956) used in these experiments, however, indicates the presence or absence of specific antigens, but does not give information on the number of antigen-antibody systems involved, the location of the antigens and the relation between morphological and chemical differentiation.

The present work was undertaken to study the formation of lens antigens during development and to correlate their appearance with that of morphological structures. Use was made of the agar-diffusion technique of Ouchterlony (1949, 1953), which is based on the differential diffusion of proteins present in a mixture.

MATERIALS AND METHODS

Antigens

Lens extracts of White Leghorn chick embryos of the following six stages were used: (*a*) 10–18 somites; (*b*) 50 hours (19–24 somites); (*c*) 60 hours (28–32 somites); (*d*) 72 hours (35–37 somites); (*e*) 96 hours (43–44 somites); and (*f*) 10 days. In addition, lens extract of new-born and adult chicken was prepared.

The lenses of the embryos were dissected and carefully cleaned from adherent iris epithelium. In stages (*a*) and (*b*) it was impossible to remove all eye-cup cells from the lens placode, due to adhesion between the two structures. The lens tissues of each group was weighed wet and homogenized to a concentration of 100 mg. wet weight/ml. in sterile 0·9 NaCl solution. In the 10–18-somite and 50-hour embryos minimal amounts of placode tissue were obtained and only a few drops of 0·9 NaCl solution were added during homogenizing.

Antibodies

A 10 per cent. lens extract prepared from adult chicken lenses was injected intravenously into healthy rabbits. The extract was administered at the rate of one injection of 3 ml. daily for 14 days. At the time of the fourth injection 1 ml. was given subcutaneously half an hour prior to the intravenous dose to avoid anaphylactic reactions. Ten days after the last injection the rabbits were bled under sterile conditions and the serum prepared. The titer of the antisera was determined by serial dilutions of lens extract and found to vary from 1:6,400 to 1:16,000. The organ specificity of the lens antiserum was tested with extracts from a variety of organs and tissues of adult chicken. A precipitin reaction was noted with extracts of iris, retina, cornea, and vitreous body, but not with other organs or tissues (Langman *et al.*, 1957).

Gel diffusion technique

The double diffusion technique of Ouchterlony (1949, 1953) was modified as follows. Agar plates were prepared with 2 per cent. dialysed filtered agar (Difco; B 140; pH 7·2) to which 0·01 per cent. merthiolate had been added (Eli Lilly & Co., solution No. 45). Ten ml. of this agar-merthiolate solution (at 85° C.) were pipetted into 15–95-mm. Petri dishes to cover the slightly curved bottoms and allowed to solidify. Another 15 ml. of the solution was poured over this layer and two plexiglass moulds were placed in the warm solution at 12 mm. from each other. After the agar had solidified the moulds were removed, thus leaving two wells referred to as the 'central' basin (10 × 10 × 3 mm.) and the 'peripheral' basin (10 × 5 × 3 mm.). The central basin was filled with lens antiserum and the peripheral one with lens extract. Occasionally four peripheral basins, each one of them located opposite a side of the central basin, were fashioned in the solidifying layer, to test lens extracts from embryos of various ages in a single agar plate. As pointed out below, precipitin bands are formed between the peripheral and central basins when critical concentrations of the diffusing antigen and corresponding antibody meet. The agar plates were examined daily and the appearance of precipitin lines recorded.

RESULTS

One to seven precipitin bands, identified by means of numbers, became visible between the central and peripheral basins in the course of the diffusion test. When a given band appeared to split into other bands, letters were added after the numbers. For each age group a photograph of the bands appearing between the peripheral and central basin (Plate 1, figs. 1*a*, 2*a*; Plate 2, figs. 3*a*–7*a*) is shown with the histological picture of the lens or lens primordium at the stage under consideration (Plate 1, figs. 1*b*, 2*b*; Plate 2, figs. 3*b*–5*b*). For the 50-hour and 60-hour embryos two histological pictures (Plate 1, figs. 1*b*, 1*c*, and 2*b*, 2*c* respectively) represent the morphological appearances that may be seen in each of these groups.

10–18-somite embryos. An extract made from presumptive lens ectoderm and eye-cup cells from 100 embryos was tested against lens antiserum. No precipitin bands were formed between the peripheral and central basin.

50-hour embryos (19–24 somites). The lens primordium is at the placode stage (Plate 1, fig. 1*b*) and the most advanced embryos in this group show acidophilic fibres in the cytoplasm, as the formation of the lens vesicle begins (Plate 1, fig. 1*c*). An extract prepared from 100 embryos and tested against lens antiserum gave rise to one precipitin band referred to as band 1 (Plate 1, fig. 1*a*). In two of the experiments an additional faint band (band 2) became visible in front of band 1.

60-hour embryos (28–32 somites). The lens vesicle invaginates (Plate 1, fig. 2*b*) and closes (Plate 1, fig. 2*c*). At this stage basophilic granules appear at the base of the cell, while the acidophilic fibres become longer. An extract prepared from the lens vesicles of 60 embryos, and tested with lens antiserum, showed three precipitin bands by the 3rd day of the diffusion test (bands 1, 2, and 3). After 4 days the bands broadened and a fourth band became distinguishable (band 4, see Plate 1, fig. 2*a*).

72-hour embryos (35–37 somites). At this stage of development the retina-facing cells of the lens vesicle show nuclear fibre formation. A lens extract prepared from 50 embryos and tested with lens antiserum revealed 4 distinct precipitin bands (bands 1–4; Plate 2, fig. 3*a*).

96-hour embryos (42–45 somites). As lens fibres become visible in the marginal zone, a lens extract prepared from 50 embryos showed the formation of 5 distinct precipitin bands when tested with adult lens antiserum (bands 1, 2, 3*a*, 3*b*, and 4; see Plate 2, fig. 4*a*).

Ten-day embryos. As lens fibres reach a large size (Plate 2, fig. 5*b*), a lens extract from 20 embryos tested against adult lens antiserum showed band 3*c* in addition to those found earlier (Plate 2, fig. 5*a*).

New-born. Lens extract of new-born chicken tested against lens antiserum usually, but not always, showed one additional band (band 5, Plate 2, fig. 6*a*), thus bringing the total to 7 bands.

Adult. Lens extract prepared from lenses of young adults always yielded a spectrum of 7 bands (Plate 2, fig. 7*a*).

Comparison of the 50-, 60-, 72-, and 96-hour lens extracts

When the 50-, 60-, 72-, and 96-hour lens extracts were respectively pipetted into one of the four peripheral basins to be tested against lens antiserum, a band 1 appeared on each side of the central basin. These bands fused at their extremities, thus outlining a square. Similarly, three bands 2 formed by the 60-, 72-, and 96-hour lens extracts coalesced. Bands 3–4 from 72-hour lens extract fused with bands 3–4 from 96-hour lens extract.

Comparison of the 96-hour, 10-day, new-born, and young adult lens extracts

Bands 1, 2, 3*a*, 3*b*, and 4, appearing on each side of the central basin and caused by the various extracts used, formed squares around the centre. Band 3*c* formed by testing 10-day lens extract coalesced with band 3*c* caused by new-born and young adult lens extracts. Band 5 formed by new-born lens extract fused with band 5 obtained by testing the lens extract from young adult animals.

DISCUSSION

The various components of an antigen mixture migrate into an agar gel at different speeds, depending on a diffusion coefficient which is determined by molecular weight, shape of the molecules, and concentration. In the 'double diffusion' test of Ouchterlony (1949, 1953) the antigens moving away from the peripheral basin are met by antibodies which migrate from the central basin. When critical concentrations of an antigen and the corresponding antibody meet, a precipitin band is formed. Neither antigen nor antibody can diffuse beyond the precipitin zone, which acts as a virtual barrier to this particular antigen-antibody system, but other antigens and antibodies go through (Ouchterlony, 1949, 1953; Wilson & Pringle, 1955; Korngold, 1956). Large differences in the concentrations of antigen and corresponding antibody will result in changes of the shape of the originally formed precipitin band. With increasing concentration the band will at first increase in density and later grow in the direction of the least concentrated component. When the precipitate is soluble in an excess of one of the components, the band moves by dissolution and reprecipitation in the direction of the less concentrated component. When a mixture of several antigens and antibodies is used, as in our experiments, a precipitin spectrum may be seen, each band corresponding to an antigen-antibody pair. Coalescence of bands, observed when various extracts are tested in one agar plate, indicates identity of antigens causing the bands.

10–18-somite embryos

In previous experiments (Langman, 1959) presumptive lens ectoderm cells were cultured in a medium containing lens antibodies. Those explants obtained from 11-somite and older embryos showed degeneration within 24 hours, indicating the presence of cellular substances able to react with lens antibodies. Nevertheless, an extract from presumptive lens ectoderm cells from embryos of this age failed to produce any precipitin band. Perhaps the concentration of lens antigen in presumptive lens ectoderm extract from 11–18-somite embryos was not high enough to reach the critical value necessary to form a visible precipitin band in the agar plate.

50-hour embryos (19–24 somites)

The extract prepared from lens placodes of 50-hour embryos and tested with adult lens antiserum showed at least one distinct precipitin band (band 1, Plate 1, fig. 1*a*) and occasionally a faint second band. This 'lens' extract, however, was contaminated by eye-cup cells. To test the possibility that a band is caused by this impurity, an experiment was made with an extract of eye-cup cells without lens tissue obtained from 50–55-hour embryos. This extract, tested in various concentrations with lens antiserum, did not show any precipitin band in the agar plates. Thus, the precipitin band(s) found with the lens extract of 50-hour embryos were caused by antigens located in the placode cells.

A problem was raised by the occasional occurrence of band 2 in front of band 1 at this stage. Two explanations may be considered. In some cases two or more precipitin lines might fuse into one. The two antigens should then have the same diffusion coefficient and the same concentration, and each antigen-antibody system should precipitate in the same critical concentrations (Korngold & Lipari, 1956). In order to investigate this possibility two different concentrations of 50-hour 'lens' extracts were tested with the same lens antiserum. In both cases the number of bands remained the same (either one or two) indicating that fusion of bands was not involved. Another possible explanation is that, in some extracts, the mean number of somites of the embryos was higher than in others. By counting the somites of the 50-hour embryos used it was found that band 2 appeared when the 'mean somite age' was closer to 24 than to 19 somites. Thus band 2 is in all probability due to the formation of a second lens antigen at the 22–24-somite stage.

It is thought that band 1 in these experiments is formed by a lens antigen located in the placode cells. The appearance of band 2 is presumably caused by a second lens antigen located in the retina-facing cells of the invaginating placode. Acidophilic fibres appearing in the apical cytoplasm of the invaginating lens placode cells are considered to be a morphological indication of the formation of this second antigen.

60- and 72-hour embryos (28–37 somites)

During this period the fibres in the cytoplasm of the retina-facing cells become longer and basophilic granules appear at the base of the cells. In addition, the cells show elongation and the first nuclear lens fibres develop. These changes coincide with the appearance of bands 2, 3, and 4, indicating the formation of substantial amounts of three lens antigens. Hence, while band 1 was considered to be a lens placode antigen, bands 2, 3, and 4 are presumed to be associated with the formation of nuclear lens fibres and would be due to lens antigens arising in these fibres. By testing a lens extract from 80-hour embryos, the same number of precipitin bands as in 72-hour lens extract was found, indicating that the number of lens antigens did not change during growth of the fibres.

96-hour embryos (42–44 somites)

At this time of development elongation of the nuclei, mitotic activity, and the appearance of acidophilic fibres and basophilic granules in the cells of the marginal zone are the most characteristic morphological features. Fig. 4*a* of Plate 2 indicates that band 3*a* is formed during the 80-96-hour period. The lens antigen causing this band is presumably located in the cells of the marginal zone and is considered to be one of the marginal lens fibre antigens.

Ten-day embryos; new-born; young adults

During this period of development fibre formation in the marginal region of the lens is in full progress. Figs. 5*a*, 6*a*, and 7*a* of Plate 2 show that some preexisting bands become dense and broad, while other new bands (3*c*–5) appear, indicating increase in concentration of some of the existing antigens and formation of new antigens. As can be seen in figs. 5*a*–7*a* of Plate 2, band 2 in particular becomes dense and broad with increasing age of the chicks providing the lens extracts. This may be due to increase in concentration of the particular nuclear lens fibre antigen. Another explanation, however, is that a new source of the same antigen is formed by the equatorial lens fibre cells. This would indicate the possibility that 'nuclear' and 'marginal' lens fibres have some antigens in common, while others are specific either for the nuclear lens fibres or for the marginal lens fibres. The antigens causing band 3*c* and 5 are considered to be representatives of the marginal lens fibre antigens.

The coalescence of bands, observed when lens extracts of various ages were tested against lens antiserum in one agar plate, indicates identity of antigens causing the bands (Ouchterlony, 1949, 1953; Wilson & Pringle, 1955; Korngold, 1956). Thus band 1 appearing in 50-hour lens extract (Plate 1, fig. 1*a*) is caused by the same antigen as in 60-hour and the other lens extracts (Plate 1, fig. 2*a*; Plate 2, figs. 3*a*–7*a*). This means that antigen 1, considered as a representative of the lens placode cells, is still present in the adult stage, though at this time it is presumably located in the anterior epithelial cells of the lens. Band 2, which occasionally appears in 50-hour lens extract and becomes clearly visible in 60-hour lens extract and dense and broad in extracts of older ages, is also caused by an identical antigen in the various extracts. The same holds for other coalescing bands.

Ten Cate and Van Doorenmaalen (1950), testing lens extracts of embryos of various ages with adult lens antiserum, obtained a precipitate as early as 60 hours and felt that they had demonstrated the appearance of 'adult' lens antigens at that stage. However, in view of Landsteiner's (1947) finding that the specificity of antigenic structures may be due to a relatively small chemical group present within a large molecule rather than to a single whole molecule, the question was raised (Langman, 1959) whether the antigens detected in 60-hour lens extract were identical to those found in adult lenses. According to the results of the present experiments, antigens present at the 60-hour stage are identical with those present in the adult stage, so that the suggestion of the above authors was correct. They were, however, only able to demonstrate the presence of some of the antigens characteristic of the adult stage, but not of all.

Since the appearance of a precipitin band in the agar plate requires a critical concentration of an antigen and a corresponding antibody, the possibility has to be considered that in the first stages of lens development all antigens characteristic for the adult stage are present, but in too low concentrations to reach the critical level necessary for the formation of visible precipitin bands. This possibility is considered unlikely, since the formation of new precipitin bands coincided with the appearance of new morphological features indicating formation of new proteins. A second argument against this possibility is that the use of increased concentrations of the various extracts did not reveal the formation of new bands.

The density of a precipitin band and its distance from the peripheral basin depends on molecular weight and concentration of the antigen molecule. The density of band 2 in 60- and 72-hour lens extract and its location in front of band 1 (Plate 1, fig. 2*a*; Plate 2, fig. 3*a*) indicate that its concentration is higher than that of antigen 1. At the 50-hour stage, however, band 2 is absent or barely visible, presumably due to a low concentration of the antigen. Even though barely visible, however, it is located in front of band 1 and it is therefore presumed that the molecular weight of antigen 2 is smaller than that of antigen 1. The same may be true for all other bands arising nearer to the antibody basin than bands 1 and 2. This would indicate that the molecular weight of antigens arising during the later stages of development is smaller than that of the first formed antigens. Evidence for this hypothesis may be found in the experiments in which François *et al.* (1955) determined the molecular weight of cow lens antigens by light scattering. It was found that the molecular weight of lens proteins in the nuclear part of the lens (bands 2, 3, and 4 in Plate 2, fig. 3*a*) was higher than that of later formed antigens. The nuclear lens antigens were so large that they were easily seen with the electron microscope (diameter about 400 A). The molecular weight of nuclear α crystallin was 200,000, while the molecular weight of later formed antigens was about 60,000. It is thus confirmed that those lens antigens appearing early in development have high molecular weights, while those appearing later have low molecular weights.

The lens antigens tested above must be water-soluble since the tests were performed with aqueous extracts. The main fractions of the lens soluble in water are known to be three holoproteins; two globulins (α and β crystallin) and an albumin-like fraction (γ crystallin) (Krause, 1932, 1933 *a*, *b*; Schaeffer & Murray, 1950; François *et al.*, 1955, 1956). Another fraction called albuminoid is non-soluble. In addition to these holoproteins Krause (1933) detected a trace of mucoprotein in the lens. In experiments on the distribution of periodic acid-reactive carbohydrates using the Schiff reagent in the adult rat, Leblond (1950) was able to find a strong reaction in the capsule of the lens, but not in lens fibres. When, however, the Schiff reagent was replaced by periodic acid-benzidine and periodic acid-dianisidine, an intense staining of the crystalline fibres of the lens was found, indicating the presence of a carbohydrate with a special type of 1,2-glycol group (Glegg, Clermont, & Leblond, 1952). To exclude the possibility that one of the precipitin bands found was caused by the lens capsule, an aqueous extract of this tissue was prepared and tested with lens antiserum. No precipitin bands were formed, indicating that the lens capsule had no soluble antigens in common with the lens itself. The possibility still remains that not all precipitin bands are formed by holoproteins of the lens, and that they may be partially formed by mucoproteins (Krause, 1933) or carbohydrates (Glegg, Clermont, & Leblond, 1952).

SUMMARY

1. The double diffusion technique of Ouchterlony, based on the differential diffusion of a mixture of antigens in an agar gel, was used to study the formation of lens antigens during development of the chick embryo and to correlate chemical and morphological differentiation. Aqueous extracts prepared from lenses of 50-, 60-, 72-, and 96-hour, and 10-day embryos, and from new-born and young adult chickens, were tested with antiserum prepared with 10 per cent. adult chicken lens extract.

2. The gradual appearance of 1–7 precipitin bands, indicating the corresponding formation of substantial amounts of up to 7-lens antigens, was noted during embryonic development. The number of bands formed was dependent upon the age of the embryos providing the lens extracts. The first appearing lens antigen causing a precipitin band was found in 50-hour lens extract (18–24 somites).

3. The appearance of new precipitin bands in the course of development, indicating the formation of new lens antigens, preceded or coincided with the appearance of morphological structures indicating synthesis of new protein.

4. Precipitin bands formed during the first stages of development (bands 2, 3, and 4 in Plate 1, fig. 2*a* and Plate 2, fig. 3*a*) were caused by antigens identical to those present in the later stages (Plate 2, figs. 5*a*, 6*a*, 7*a*).

5. The molecular weight of the first formed antigens appeared to be higher than that of the antigens formed later in development.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1*a*. Precipitin band 1 found by testing 50-hour 'lens' extract with lens antiserum.

1*b*. Lens placode from 19-somite embryo; cells have cylindrical shape and nuclei are located perpendicularly to retinal surface.

1*c*. Invaginating lens placode from 24-somite embryo; acidophilic fibres appear in cytoplasm of the cells (see arrow).

FIG. 2*a*. Precipitin bands 1, 2, 3, and 4 found by testing 60-hour 'lens' extract with lens antiserum.

2*b*. Invaginating lens placode from 28-somite embryo; acidophilic fibres become longer (see arrow).

2*c*. Lens vesicle is completed but remains attached to ectoderm in 32-somite embryo. Retina facing cells stain more acidophilic and become elongated.

PLATE 2

FIG. 3*a*. Precipitin bands 1, 2, 3, and 4 found by testing 72-hour lens extract with lens antiserum.

3*b*. Lens vesicle from 36-somite embryo shows beginning nuclear lens fibre formation.

FIG. 4*a*. Precipitin bands 1, 2, 3*a*, 3*b*, and 4 found by testing 96-hour lens extract with lens antiserum.

4*b*. Lens from 44-somite embryo; nuclear lens fibres have filled the lumen of the lens vesicle and the cells in the marginal zone show beginning fibre formation.

FIG. 5*a*. Precipitin bands 1, 2, 3*a*, 3*b*, 3*c*, and 4 found by testing 10-day lens extract with lens antiserum.

5*b*. Lens from 10-day old embryo showing nuclear- and marginal lens fibres.

FIG. 6*a*. Precipitin bands 1, 2, 3*a*, 3*b*, 4, and 5 found by testing new-born lens extract with lens antiserum.

FIG. 7*a*. Precipitin bands 1, 2, 3*a*, 3*b*, 3*c*, 4, and 5 found by testing young adult lens extract with lens antiserum.

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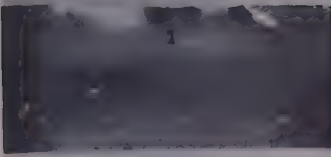

fig. 1a

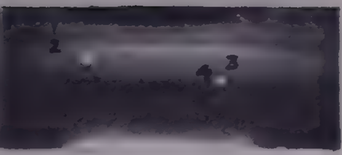

fig. 2a

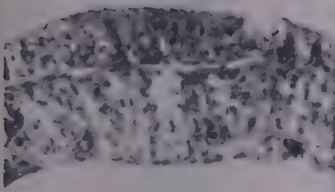

fig. 1b

19 som

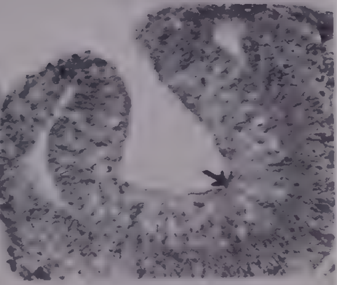

fig.2b

28 som

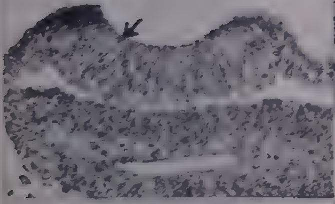

fig. 1c

24 som

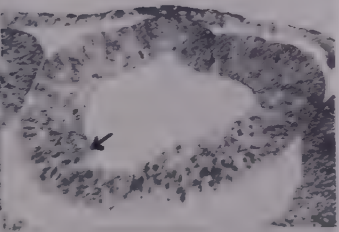

fig. 2c

32 som

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Plate 1

72 hr

96 hr

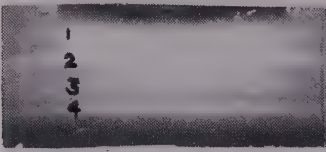

fig. 3a

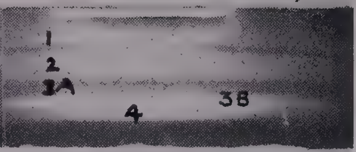

fig. 4a

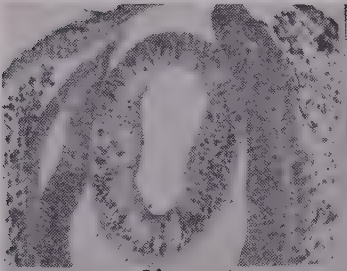

fig. 3b

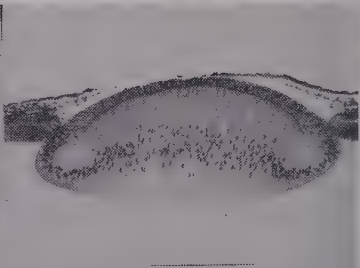

fig. 4b

36 som

44 som

10 day

new born

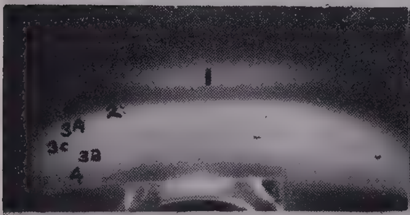

fig. 5a

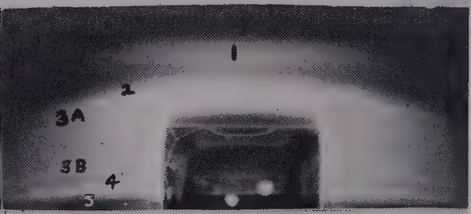

fig. 6a

young adults

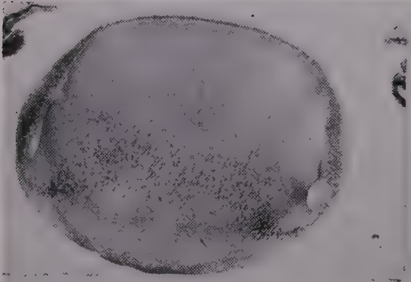

fig. 5b

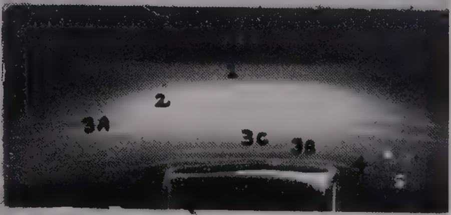

fig. 7a

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Plate 2

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